

EVALUATION OF THE PARASITIC RELATIONSHIP OF *MELANOSPORA* AND
OTHER ALLIED GENERA WITH *FUSARIUM OXYSPORUM*

By

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Fusarium wilt, caused by *Fusarium oxysporum*, has been a problematic disease for many different crops for over 100 years. The discovery of a group of pyrenomycetous ascomycetes found naturally in association with several *formae speciales* of *F. oxysporum* from field samples, has generated the interest to investigate the use of these fungi as biocontrol agents for watermelon wilt caused by *F. oxysporum* f. sp. *niveum* (FON). The first of these fungi was isolated from the sugar beet pathogen, *F. oxysporum* f. sp. *betae*, and was identified as *Melanospora zamiae*. Later findings from other wilt-infected crops yielded another five isolates that very closely resembled the original *M. zamiae*. However, based upon current taxonomic criteria, these five isolates were determined to consist of two distinctly different genera, *Sphaerodes retispora* var. *retispora*, and *Persiciospora moreau*.

All six isolates were found to be strongly dependent upon *F. oxysporum* for growth and sporulation. Linear growth and production of perithecia were significantly

improved for all isolates in dual culture with *F. oxysporum* or growing in filtrate from a liquid culture of *Fusarium*. Significant differences in growth were observed among and between the different pyrenomycetes. Without the presence of *F. oxysporum* or its metabolic growth products, growth and reproduction were generally very poor for all isolates. The better media without *Fusarium* tended to be those composed of simple sugars like dextrose or maltose, while those producing the worst results contained starch or cellulose. All isolates were determined to be parasitic on *F. oxysporum* through the use of several cultural assays, but not all were pathogenic to FON. *Persiciospora* caused no detectable reduction in hyphal growth, whereas the *Sphaerodes* isolates were the most harmful.

Isolate LE (*S. retispora*) was selected for disease reduction evaluations in the greenhouse. It was incorporated into sodium alginate pellets and delivered to the infection court by either root treatment or amendment to infested soils before transplantation. Yield data were collected after eight weeks, and consisted of mortality counts and total plant dry weights. This technique did significantly improve plant dry weights of treated plants compared to the FON-inoculated controls. These are promising results for an alternative method for managing Fusarium wilt of watermelon. However, it is not known whether this procedure is efficacious or even feasible under field conditions. If proven to be successful in the field, this procedure could also presumably be utilized for disease control with other vegetable crops in Florida that are similarly grown as transplants. However, care must be taken for the selection of the potential biocontrol isolates, because not all included in this study were found to equally colonize or induce deleterious effects upon all *formae speciales* of *F. oxysporum*.

CHAPTER 1 INTRODUCTION

The genus *Fusarium* was first erected in 1809 by Link when he described *F. roseum* as the first species (Booth 1984). *Fusarium* species are ubiquitous soil fungi found in many climatic extremes such as deserts and the arctic, in addition to temperate and tropical regions of the world. They can be saprophytes and pathogens infecting both animals and plants (Nelson 1981). Because of variation in culture, it has been difficult for workers to correctly classify this genus into species. The basis for *Fusarium* classification was established by Appel and Wollenwebber (1910). They placed species studied into sections based on various morphological characteristics (Coons 1928). These sections were later named and expanded to include a type species for each section (Wollenwebber 1913).

Of the 43 species of *Fusarium* listed by Booth (1971), 27 are pathogenic to plants and cause some of the most important diseases in agriculture. The pathogens may be divided into three main categories: the wilts, the root rots caused primarily by *F. solani*, and those fungi attacking graminaceous plants, such as *F. moniliforme*, *F. graminearum*, *F. culmorum*, and *F. avenaceum* (Price 1984). The vascular wilts, caused by *formae speciales* of *F. oxysporum*, are considered to be the most economically important due to the diversity of plants attacked and the destructiveness of the diseases (Nelson 1981).

Fusarium oxysporum was the first pathogenic species described, and was included in the section *Elegans* by Wollenwebber and Reinking (1935). They divided this section into three subsections and further subdivided these subsections into 10 species, 18 varieties, and 12 forms (Nelson 1981). Their taxonomic system was difficult to use by others because they placed such great emphasis on spore measurements and septations, which are highly variable.

Snyder and Hansen (1940) simplified Wollenwebber and Reinking's system by combining all members of the section *Elegans* into one species, *F. oxysporum*, by the utilization of standard culture media and incubation temperatures. The different parasitic forms were recognized by their selective pathogenicity to specific crops and were designated as *formae speciales*. *Formae speciales* are also further subdivided into biological races based on pathogenicity to differential cultivars containing different resistance genes.

Recognition of the limiting effect of the *Fusarium* wilts on crop production goes back to the 1890's. Atkinson (1892) described the first *Fusarium* wilt disease, and referred to it as "Frenching." It had been causing serious losses to Alabama cotton growers. He demonstrated the presence of gummy substances blocking the vascular elements and illustrated the phialids producing the microconidia, which has proven to be the main diagnostic criterion for identification of *F. oxysporum*. He also named the causal agent as *F. vasinfectum* (Atkinson 1892), which is now known to be the form name for *Fusarium* wilt of cotton. There are many additional early examples of crops threatened by *Fusarium* wilts including potatoes, celery, tomatoes, flax, bananas, cabbage, cowpea, and watermelon.

The cultivated watermelon, *Citrullus lanatus* (Thunb.) Matsum and Nakai, is the most economically important member of the genus *Citrullus*, and is grown for fresh consumption as a desert vegetable. The citron, *C. lanatus* var. *citroides* (L. H. Bailey) Mansf., is not eaten fresh, but is candied. It has also been important in breeding for disease resistance because it readily crosses with the watermelon, and is resistant to Fusarium wilt. Watermelons are an important truck crop in the South. Commercial production is approximately 184,000 acres nationally, concentrated in Florida, Texas, Georgia, and California (Peirce 1987). It has an estimated gross market value of \$150 million (Martyn 1986).

The watermelon is considered to be native to the southern deserts of Africa. David Livingstone reported that wild populations covered vast areas of the Kalahari Desert after unusually heavy rains. Both natives and animals were observed consuming the fruit (Livingstone, 1859). Carrier, however, concluded wrongly that it was indigenous to North America based on descriptions by French explorers of Indians growing melons in the Mississippi Valley (Carrier 1924).

The actual time and place of domestication of the watermelon is unknown, but by 2000 B.C. it was being cultivated along the Nile by Egyptians and widely grown by prehistoric agricultural peoples of sub-Sahara Africa (Peirce 1987, Sauer 1994). Watermelons were introduced to India and China by A.D. 1100, and brought to Europe by the Moors. Popularity in Europe spread slowly, presumably because of the long, hot growing season required for good yields (Sauer 1994). In North America, Spanish colonists were growing them in Florida during the 1500's, and by 1650, Dutch and British colonies in the Northeast were cultivating them abundantly (Peirce 1987). Watermelons

were enthusiastically adopted by the North American Indians. By 1700, they were being grown from the Great Lakes region to the American southwest and lower Rio Grande Valley of Texas (Sauer 1994). This probably explains Carrier's mistaken conclusion that they originated in North America. Hawaiians also began growing watermelons after being given seeds by Captain Cook on one of his voyages (Sauer 1994).

Fusarium wilt of watermelon was first described in 1894 from Georgia and South Carolina by E. F. Smith (Smith 1894). By the time of his second report (Smith 1899), it had been identified throughout the melon-growing areas of the Southeast ranging from Texas to Maryland. Taubenhaus began investigations on this disease in Texas in the late teens because it was considered to be the most important and destructive disease affecting watermelons (Taubenhaus 1920). He showed that the organism was widely distributed at different soil depths, that disease became more severe and spread more rapidly after using manure than with commercial fertilizers, and that activity of the pathogen was greater under warm conditions than cool. He further concluded that after a three year absence of melons in fields, pathogen levels were reduced, but not completely eradicated (Taubenhaus 1920). Elsewhere throughout the South, large tracts of land, previously cropped profitably to watermelons, were rendered unfit and abandoned because of this disease. It is now known to be widespread throughout the U.S. and wherever else watermelons are grown, and is considered to be the primary limiting factor to production in many of these areas.

Smith's (1899) research on the watermelon wilt was combined with a wilt of cowpea and extended work on the wilt disease of cotton originally described by Atkinson. He gave the fungi causing these diseases different names: *F. nivum*, *F. tracheiphila*, and

F. vasinfectum for watermelon, cowpea, and cotton, respectively. This was done because they could not be distinguished in pure culture, but he determined that they differed in pathogenicity after cross inoculation studies on each crop host. He further observed that land infested with the melon fungus should not be planted with watermelons, but could be grown with cotton or cowpea. He also demonstrated that the pathogen could be spread to new land on seeds, in run-off water, barnyard manure, and in soil clinging to farm equipment (Smith 1899).

These outstanding researches were marred however, by Smith's erroneous conclusion that *Neocosmospora*, found on dead stems, was the teleomorph of the Fusarium wilt pathogen (Smith 1899). This conclusion caused some confusion until it was first challenged by Higgins in 1909 (Higgins). He proved that *Neocosmospora* was a saprophyte and distinct from the organism causing the wilt disease of watermelon. This was subsequently confirmed by others (Taubenhaus 1920, Wollenwebber, 1913).

The pathogen survives in soil primarily as chlamydospores and may remain dormant for many years. This stage serves as the predominant source of inoculum although infection may also occur via macro- and microconidia (Martyn 1996). Chlamydospores may germinate in response to host or nonhost roots, or by contact with fresh noncolonized plant debris (Nelson 1981). After chlamydospore germination, conidia and new chlamydospores may be formed, in addition to hyphae. Penetration then occurs on the host plant either directly or through wounds.

Once the pathogen enters a susceptible host, it invades the vascular system where it becomes systemic and moves throughout the plant by means of hyphal growth or movement of microconidia in the vascular stream (Martyn 1996, Nelson 1981). It is

generally confined to the xylem vessels and tracheids initially, but may also enter the parenchyma later. As disease development progresses, the fungus may also move into adjacent tissues such as the pith, cambium, phloem, or cortex (Nelson 1981). At this point wilt symptoms are severe and often result in plant mortality. After the plant dies, chlamydospores are reformed from mycelium or macroconidia and serve as the overwintering propagule. In resistant plants, colonization is generally limited to the cortex and several vessel elements (Martyn 1996).

Fusarium oxysporum f. sp. *niveum* has been shown to be seedborne in low levels, however the major mechanisms of spread are via movement of infested soil or plant debris. The disease is usually more severe on light, sandy soils with a low pH, and has an optimum soil temperature requirement between 20-27°C (Martyn 1996).

Control of this disease is very difficult to achieve. It was noted long ago that spraying plants with Bordeaux mixture (CaSO₄ and lime) or a similar fungicide had little or no effect because of the location of the fungus inside the vascular system (Taubenhaus 1920). Control with broad spectrum soil fumigants is erratic and expensive (Martyn 1986). However, it has been shown to be effective under certain conditions. Hopkins and Elmstrom (1979) successfully obtained commercial yields on severely infested lands in Florida utilizing sodium azide and chlorinated hydrocarbons combined with methyl isothiocyanate (DD-MENCS). This was only possible with moderate or highly resistant cultivars, but not with susceptible ones. They further demonstrated that control was more consistent and effective when applications were made prior to bed formation due to deeper penetration of the chemicals (Hopkins and Elmstrom 1979). However, some

studies since this time have provided evidence that recolonization of *Fusarium* after fumigation still can occur in some cropping systems (Marois and Mitchell 1981).

Biological control is another form of disease management that is becoming increasingly popular and important for *Fusarium* wilt control. Cook and Baker (1983) define biological control as "the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man". Their very broad-based definition includes cultural practices, breeding for resistance, or the use of antagonists, nonpathogenic, or other beneficial microorganisms either introduced or resident (Cook and Baker 1983).

Numerous cultural methods that attempt to modify the environment to the advantage of the host have proven to be of some value for *Fusarium* wilt management. Avoiding monoculture of watermelons is one of the simplest and most important of these methods. In most cases, continuous culture tends to increase inoculum potential of soils. An exception to this was noted with the use of the moderately resistant cultivar Crimson Sweet. After four-five years, it induced a wilt suppressive soil (Hopkins and Elmstrom 1984, 1987). Because of the capacity of *Fusarium* to survive as chlamydospores or on roots of plants as symptomless carriers, control through crop rotation alone appears to be unlikely. Rotation also should be combined with long periods (five-seven years) without growing watermelons. Planting melons on new land is another excellent strategy to avoid wilt, however, this is also very impractical for many producers. Soil solarization is another cultural method that has shown promise by delaying onset of wilt and reducing overall disease incidence. It reduced initial inoculum and overall population levels in soil,

but was only effective in fields with moderate to low inoculum levels (Martyn 1986, 1996).

The management of crop nutrition has also resulted in varying levels of success in controlling Fusarium wilt diseases. These diseases have been demonstrated to be less severe in soils with a high pH. Since the addition of many elements profoundly influences soil pH, it is not known whether the pH itself or the increased plant nutrition is responsible for wilt control. One possible explanation for this phenomenon has been hypothesized that at high pH, many micronutrients tend to become unavailable. *Fusarium oxysporum* has a high requirement for these elements, and its inability to grow and sporulate under these conditions may explain reduced disease at high pH (Jones et al 1989). Evidence for this idea was obtained when tomato wilt still occurred in limed soils in Florida after certain combinations of trace elements (iron, manganese, and zinc) were applied (Jones and Woltz 1981). Regardless of the mechanism responsible for reducing disease severity, Jones et al. (1989) demonstrated that the use of lime, $\text{NO}_3\text{-N}$ instead of $\text{NH}_4\text{-N}$, and low phosphorus was highly effective for Fusarium wilt of tomato. However, Hopkins and Elmstrom (1976) found no significant differences in watermelon wilt control comparing the nitrogen fertilizer treatments of NO_3 and NH_4 .

The most effective control measure for any Fusarium wilt disease, other than long rotations and planting on new land, is generally considered to be the use of resistant cultivars. The first known instance of developing a plant variety with resistance to a Fusarium disease by hybridization was for watermelon wilt. Orton (1907, 1909) was able to create the wilt resistant cultivar Conquerer by crossing the inedible but resistant citron melon with the susceptible cultivar Eden. Conquerer was not widely used commercially

due to certain horticultural limitations, but it was employed as a parent for early resistance breeding (Porter and Melhus 1932). Since that time, extensive breeding programs have successfully produced a number of wilt resistant cultivars, which had their beginning with this original cultivar (Walker 1957). The major disadvantage of using genetic resistance is that performance of individual cultivars may vary among different locales because of different disease reactions of pathogen races. Currently there are 3 known races of *F. oxysporum* f. sp. *niveum* (races 0, 1, and 2). Race 0 is pathogenic only to cultivars with no resistance genes. Since most modern cultivars have some *Fusarium* wilt resistance, this race of the pathogen is no longer important. High levels of race 1 resistance are available for many good commercial cultivars. Race 2 isolates have only been identified, to date, from Israel, Texas, Oklahoma, and Florida (Martyn, 1996), but will undoubtedly be problematic for watermelon growers from other locations within the near future.

Induced resistance and suppressive soils are two of the best known and successful biological control methods that utilize microorganisms. Induced resistance, also known as cross protection and immunization, has been extensively documented against all classes of pathogens in many different plant pathosystems, however, it has been best exemplified for disease control of foliar pathogens of cucurbits, beans, and tobacco (Kuc 1982, 1990).

Induced resistance has also been shown to be effective for a number of vascular wilt diseases. Reduced *Fusarium* wilt has been reported by inoculation of susceptible hosts with nonpathogenic races or different wilt pathogens prior to challenge with the target pathogen. The selection and subsequent inoculation of nonpathogenic strains of *F. oxysporum* have been shown to successfully reduce wilt by pathogenic strains in celery

(Schneider 1984), cucumber (Paulitz et al. 1987), sweet potato (Ogawa and Komada 1985), and watermelon (Biles and Martyn 1989, Larkin et al. 1996).

The inoculation of non-host pathogens has additionally been documented in reducing disease severity. Phillips et al. (1967) observed reduced wilt of tomato by inoculations of *Cephalosporium* spp. Another interesting example by Gessler and Kuc (1982) demonstrated that root protection from *F. oxysporum* f. sp. *cucumerina* on cucumber could be induced systemically with other *formae speciales* (*melonis* and *conglutinans*), or by leaf inoculation with *Colletotrichum lagenarium* or tobacco necrosis virus. Specifically, watermelon wilt has similarly been proven to be effectively reduced by preinoculation with the non-watermelon pathogens *F. oxysporum* f. sp. *cucumerina* (Biles and Martyn 1989) and *F. oxysporum* f. sp. *lycopersici*, *Verticillium albo-atrum*, and *Helminthosporium* (*Cochliobolus*) *carbonum* (Shimotsuna et al. 1972).

Conversely, Wymore and Baker (1982), concluded that using this approach to wilt control of tomatoes under greenhouse conditions was not practical. The induced resistance initially obtained required inoculum densities of the cross protection agents to be of equal or greater than that of the challenge pathogen. Additionally, the protection that did occur was only temporary by delaying symptoms and eventually produced disease equal to that of the inoculated controls (Wymore and Baker 1982).

Over the last 25-30 years, the greatest progress achieved in biological control employing soil microorganisms has been investigating and understanding the principles responsible for pathogen suppressive soils (Cook 1990). Most soils are suppressive some degree, but those that are pathogen suppressive have a unique ability to reduce the impact of soilborne pathogens following certain agronomic management systems. Suppressive

soils have been defined by Cook and Baker (1983) as those soils where disease never becomes established or persists, becomes established but causes little damage, or is established and causes disease for a short time but afterward becomes less problematic even though the pathogen is still detectable in these soils.

The occurrence of pathogen suppressive soils over the years has been recognized primarily because of observed disease severity in crops being lower than was anticipated (Baker and Cook 1974). In general, suppressiveness may act directly on the pathogen by affecting propagule growth and survival, or it conceivably can work indirectly through the host in the form of cross protection or induced resistance (Cook 1990, Cook and Baker, 1983). This example of induced resistance is different from the one previously described by being resident or naturally-occurring in the soil rather than being introduced.

Pathogen suppressive soils are now known for 10-15 plant diseases (Cook 1990, Cook and Baker 1983). With the possible exception of take-all suppressive wheat soils (*Gaeumannomyces graminis* var *tritici*), the most extensively studied suppressive soils worldwide are those of Fusarium wilt. The existence of wilt suppressive soils has been recognized for over 100 years, and research on them has been conducted for greater than 70 years. Atkinson (1892) first noticed that cotton wilt was more severe on sandy soils in Alabama. Later work on wilt suppressive soils from Central America involving banana wilt (*F. oxysporum* f. sp. *cubense*) and the pea wilt caused by *F. oxysporum* f. sp. *pisi* in Wisconsin similarly noted that disease severity was less devastating on heavier clay soils than on lighter, sandy ones (Toussoun 1975, Reinking and Mann 1933, Walker and Snyder 1933)

A number of other agricultural soils throughout the world exhibit a similar phenomenon of disease reduction, including several areas of the San Joaquin and Salinas valleys of California (Scher and Baker 1980, Smith and Snyder 1971), the Chateaufort region of the Rhone Valley in France (Alabouvette 1986, 1990), and on an experimental farm in Central Florida (Hopkins and Elmstrom 1984, 1987). Most of these soils have a number of characteristics in common. They tend to be alkaline and heavy-textured. Suppressiveness is destroyed by heat treatments, but can be restored by the addition of small quantities of suppressive soil. This suggests a biological origin for induction and maintenance of suppression. Suppression to a particular *forma specialis* is also suppressive to other pathotypes of *F. oxysporum*, but not to nonpathogenic *F. oxysporum* or other soil pathogens. This also holds true for other species of *Fusarium*. Pathogen suppressive soils also tend to have higher concentrations of overall soil microflora than do wilt conducive soils (Cook and Baker 1983). Competition for iron or carbon between the pathogens and resident bacteria and/or nonpathogenic fusaria is believed to be responsible for suppression in many of these soils. The mechanism of suppression in other soils seems to be a general resistance in plants induced by nonpathogenic *F. oxysporum* or growth-promoting rhizobacteria (GPR). They have the ability to infect plants before the pathogens due to a higher degree of aggressiveness.

The watermelon wilt suppressive soil from Florida is the curious exception to several of these traits characteristic of most wilt suppressive soils. It has similarly been shown to harbor higher populations of soil microorganisms than wilt conducive soils, but is different by its sandy, acidic nature and low organic matter content. This soil is also unique by suppression being induced by monoculture of the moderately resistant cultivar

Crimson Sweet (Hopkins and Elmstrom 1984, 1987). The suppressive effect of the other soils is believed to be constitutive. The primary mechanism of suppression in the Florida soil is not competition for micronutrients among native soil organisms, but an induced resistance caused by the high populations of nonpathogenic *F. oxysporum* isolates.

Mycoparasitism is another method that has been utilized in controlling soilborne pathogens, and this term is defined as the parasitism of one fungus by another (Hawksworth et al. 1995). Barnett and Binder (1973) divided mycoparasites into two major categories based upon their mode of obtaining nutrition. The highly aggressive necrotrophic parasites make contact with hosts, secrete hyphal wall-degrading enzymes causing death of the host cell, and finally utilize nutrients released through this process (Barnett and Binder 1973, Jeffries and Young 1994). Necrotrophic parasites tend to have large host ranges and are largely unspecialized as to their mechanism of parasitism. Most members of this group are opportunistic and capable of living saprophytically or competing with other organisms for space and nutrients (Jeffries and Young 1994).

The biotrophic parasites, conversely, obtain their nutrition from living host cells. The living host supports the parasite and may appear unaffected at first. Throughout the years of close association, some biotrophic parasites have lost the ability to synthesize one or more required nutrients, and must rely upon the host to supply them. Not only must the host supply these food stuffs, but the parasite must also be capable of successfully retrieving them from the host. Thus a physiological balance is established in this relationship and the parasite appears to be highly adapted (Jeffries and Young 1994). This is probably why biotrophic parasites tend to be more restrictive in host range than do necrotrophs. They also form specialized infection structures, or what Jeffries and Young

(1994) call "host-parasite interfaces." Additionally no biotrophic parasite has ever been demonstrated to produce cell-degrading enzymes or exotoxins (Jeffries and Young 1994).

There are 3 separate types of biotrophic relationships that can be distinguished based upon the morphology of this interaction. There are those in which the whole thallus of the parasite enters the hyphae of the host, and are called intracellular biotrophs. The haustorial biotrophs send a lobed haustorium into host cells via invagination of the plasmalemma. Nutrients are absorbed from host cytoplasm back to the parasite through this haustorium. The third type is apparently very unusual and the most specialized of the three. It involves physical contact between host and parasite, but no penetration occurs. Until the ultrastructural studies of Hoch (1977), it was not understood specifically how this relationship functioned. His work showed that nutrients are obtained by the parasite through the formation of channels of contact between closely appressed host-parasite hyphae. Specialized contact or buffer cells form on the parasite and contact the host hyphum without penetration. These cells serve as a stable interface for the transfer of the nutrients through channels similar to plasmodesmata (Hoch 1977). The plasma membrane of both organisms come into contact and fuse, therefore cytoplasmic continuity is achieved (Hoch 1977, Jeffries and Young 1994). Since this is such an important feature of this type of parasitism, it has been renamed fusion biotrophism, to replace the older term, contact biotrophism (Barnett and Binder 1973, Jeffries and Young 1994).

Fusion biotrophs consist primarily of anamorphic hyphomycetes, although several teleomorphic states of the Ascomata have also been reported (Jeffries and Young 1994). These include several closely related genera like *Gonatobotrys*, *Gonatobotryum*, and *Nematogonium* (Whaley and Barnett 1963, and Barnett and Binder 1973). The

pyrenomycete *Melanospora zamiae* has also been reported to parasitize a large number of fungi, (Hanlin et al 1993, Jordan and Barnett 1978), which is considered to be unusual for a fusion biotrophic mycoparasite.

Interestingly, one of the first biotrophic parasites discovered was that of *Gonatobotrys simplex* (Whaley and Barnett 1963). It also happened to serve as the model for Hoch's ultrastructural work with fusion biotrophs (Hoch 1977). The teleomorph of *G. simplex* has further been identified as *Melanospora dammosa*, which is itself a fusion biotrophic parasite of *Fusarium sacchari*, causal agent of corn stalk rot (Vakili, 1989).

Because of the unique ability of *F. oxysporum* to inhabit and colonize the specialized niche inside a plant's vascular system, no studies have been published attempting to control wilt diseases with mycoparasites that act directly on the pathogen. Cook and Baker (1983) point out that in general, the more internal a pathogen is during the host-pathogen interaction, the less vulnerable it is to antagonists because of the degree of protection afforded by the plant. The vascular wilt fungi, and *F. oxysporum* in particular, have only a brief epiphytic stage and exist almost entirely as endophytes within the root cortex and vascular tissue. However, these same authors also point out that the specialization required for this type of life strategy can also lead to an increased vulnerability to the physical environment and antagonists when not residing within the host (Cook and Baker 1983, Baker and Cook 1974).

Owing to the difficulties associated with direct observation of living fungi in the soil, most information concerning mycoparasites and their hosts have been obtained, by necessity, from dual cultures in vitro, or by inference from field observations (Jeffries and Young 1994). Mycoparasites are also notoriously difficult to observe in the lab because

of slow or poor growth in axenic culture. Very little is known about the natural occurrence of fusion biotrophic mycoparasitism, however because of these reasons, this field is wide open for exploration.

The impetus behind this research began while collecting isolates of *F. oxysporum* f. sp. *betae* from sugar beet in Texas. After several weeks, approximately 10-15% of the isolates from the field became contaminated with an unknown ascomycete that seriously affected growth and vigor of *Fusarium* in culture. This unknown fungus was identified as *Melanospora zamiae*, and was determined to be occurring naturally with *F. oxysporum* f. sp. *betae*. Over the last year, five additional isolates of Pyrenomycetous ascomycetes have been found in Florida associated with field populations of three additional plant pathogenic *formae speciales* of *F. oxysporum*. These organisms are closely related to, but distinct from, *M. zamiae*.

This study will attempt to address some of these aspects involving the mycoparasitism of *F. oxysporum* by *M. zamiae* and other allied genera. None of these additional genera have been conclusively shown to be parasitic. Chapter 2 will cover the identification and taxonomy of this group of Pyrenomycetes. Chapter 3 will address the stimulation of growth and reproduction of the parasites by the *F. oxysporum* hosts. Chapter 4 will determine host range and measurable damage caused by the parasites to *F. oxysporum* in culture. Chapter 5 will delve into some ecological aspects of the parasite and its ability to colonize both plant hosts and *F. oxysporum*, and to survive in soil. Chapter 5 will also attempt to combine knowledge from other aspects of this study and attempt to utilize one or more of these parasites as a biological control agent for watermelon wilt at the greenhouse level.

CHAPTER 2 A TAXONOMIC STUDY OF *MELANOSPORA* AND ITS ALLIES

Introduction

During the course of studying soilborne isolates of *Fusarium* causing root diseases of a number of crops, species resembling *Melanospora* were found in constant association with these organisms. These *Melanospora*-like strains appeared to be mycoparasitic and causing damage to the fusaria. Because there has been confusion as to the limits of *Melanospora*, a study of this genus and its closely related allies appeared warranted.

The genus *Melanospora* was first erected in 1837 by Corda (1837). He described three species in the following order, *M. zamiae*, *M. chionea*, and *M. leuchotricha* (Shear and Dodge, 1927). Because he did not designate a type species, most authors have considered *M. zamiae* to be the type (Von Arx 1981, Cannon and Hawksworth, 1982), although Clements and Shear described *M. chionea* as the type (1931).

Melanospora is currently classified as belonging to the Ceratostomataceae in the Sordariales (Hawksworth et al, 1995, Barr, 1990). However, this genus has long been a taxonomic problem for mycologists and has resided in at least five orders and six families since its first description. Gilmer (1957), and Gwynne-Vaughn and Barnes (1927) both included it in the Nectriaceae of the Hypocreales while Alexopoulos (1962) and Stevens (1974) placed it in the Melanosporaceae of the same order.

Numerous authors have placed *Melanospora* in the now invalid order Sphaeriales. Within this order, *Melanospora* has been included in the Melanosporaceae (Dennis 1978,

Bessey 1935, 1961, Munk 1957), Hypocreaceae (Clements and Shear 1931), Ceratostomataceae (Fitzpatrick 1934), and the Xylariaceae (Nannfeldt 1932).

Based on centrum development, Luttrell (1951) included *Melanospora* in the Melanosporaceae of the Diaporthales. This placement was established to include genera from the Hypocreales that had a *Diaporthe*-like centrum, but resembled members of the Diaporthaceae in centrum-type only.

Weymeyer (1975) recognized three families in the Melanosporales, Chaetomiaceae, Melanosporaceae, and Ceratocystaceae, and placed *Melanospora* in the Melanosporaceae. He regarded the order as intermediate between the Plectascales and the Hypocreales or Sphaeriales. Alexopoulos et al. (1996), listed the genus in the Melanosporales, but designated it as a monotypic order with no familial rank.

Over one hundred species have been named *Melanospora* (Goh and Hanlin 1994), but Doguet (1955) considered most to be synonyms. Doguet introduced a broad concept of the genus and arranged species into groups based on shape and ornamentation of the ascospores. Recently this concept of *Melanospora* has been narrowed, and many genera have now been separated from Doguet's broad treatment of the genus (Udagawa and Cain 1969, Hawksworth and Udagawa 1977, Cannon and Hawksworth 1982). Based on Cannon and Hawksworth's (1982) study utilizing scanning electron microscopy, *Melanospora* is now restricted to those fungi with smooth ascospore walls and depressed germ pores.

Of all the many synonyms used for species of *Melanospora*, the two most commonly accepted have been *Sphaeroderma* Fuckel, and *Microthecium* Corda. *Sphaeroderma* was distinguished from *Melanospora* by the absence of an ostiolar neck,

but this trait is considered to be highly variable (Cannon and Hawksworth 1982). *Microthecium* was retained for cleistothecial species, but otherwise identical to *Melanospora* (Udagawa and Cain, 1969, Hawksworth and Udagawa 1977). Scanning electron microscopy studies have now confirmed that both cleistothecial and perithecial species with reticulately ornamented ascospores constitute a distinct genus for which the name *Sphaerodes* Clem. was available (Von Arx 1981). Because species of *Microthecium* are characterized by smooth-spored ascospores with depressed germ pores, they are now synonymized with *Melanospora*, while those with reticulated spores formally placed there have been transferred to *Sphaerodes* (Cannon and Hawksworth 1982).

Sphaerodes was erected by Clements in 1909 to include *S. episphaeria*, and was distinguished from *Sphaeroderma* by the lack of a subiculum (Clements and Shear 1931). Besides the reticulated spores, *Sphaerodes* differs from *Melanospora* in the structure of the germ pores. It has protruding, tuberculate pores, while *Melanospora* has pores either level with spore surface or slightly sunken or depressed (Cannon and Hawksworth 1982).

Persiciospora is a newly created genus to retain *Melanospora*-like pyrenomycetes with yet another type of ascospore morphology. It is distinguished from *Melanospora*'s smooth spores and *Sphaerodes*' reticulated ones by producing spores with delicately pitted walls similar to a peach seed (Cannon and Hawksworth 1982).

Apart from ascospore structure and morphology, *Melanospora* and its allies, *Sphaerodes* and *Persiciospora*, are similar in numerous respects. They are characterized by superficial, nonstromatic perithecia composed of angular pseudoparenchymatous cells. Perithecia are spherical, amber to pale yellow in color and are solitary to gregarious. Four-to eight-spored, clavate asci are soon deliquescent with spores filling the perithecial

cavity and exuding out of the ostiolar neck in a slimy cirrhus. Ascospores are dark, and ellipsoid or oval-shaped. The yellowish, translucent perithecia appear black when filled with mature ascospores. Ostiolate species have long or short beaks, often ringed with hyaline setae of varying lengths. Centra are composed of thin-walled pseudoparenchyma and lack paraphyses at maturity. Many species have additionally been suspected of being parasitic on other fungi.

The primary reason for the extensive taxonomic changes for *Melanospora* is that its characteristics are not consistently applicable to all the families and orders within which it has been classified. It does not fit well within any of the ranks. It was placed in the Hypocreales because of its fleshy, unpigmented perithecium, and phialidic anamorphs (Rehner and Samuels 1995). Many species of this order, such as *Melanospora*, also have clavate asci and a presumed ecological role as mycoparasites. However, *Melanospora* differs from other members of the Hypocreales in its aseptate, dark ascospores with germ pores at each end, and deliquescent asci. *Melanospora* also lacks the apical paraphyses or pseudoparaphyses of the *Nectria*-type centrum development characteristic of this order (Rehner and Samuels 1995).

There has never been a standard, universally recognized classification for the Sphaerales. It once included all pyrenomycetes, but the Hypocreales, Clavicipitales, and Laboulbeniales were removed based on centrum development and ascal characteristics (Luttrell 1951). The remainder constituted the single, heterogeneous order Sphaerales, which did not fit well within any other order of the Euascomycetes (Wehmeyer 1975). Therefore, it is no longer recognized as an acceptable taxonomic rank. Members of this old order were characterized by clavate or cylindric asci, that were borne in a hymenial

layer. Asci opened by a round, apical pore, and were forcefully ejected. They additionally contained haematecia consisting of varying numbers of paraphyses (Alexopoulos 1962). *Melanospora* and its allies were considered members of this catch-all group probably because of their varied, hybrid-like characteristics. Since they had to be placed somewhere, this was considered to be the best location. They were similar in the possession of clavate asci borne in a true perithecium, but were different by lacking the paraphyses or poroid asci.

Luttrell (1951) placed *Melanospora* in the Diaporthales based on similar early perithecial development. However, this group is different from *Melanospora* in almost every other respect, including persistent, poroid asci with a refractive ring at the apex, and ascal development at several levels within the perithecium (Alexopoulos et al. 1996).

The current placement of *Melanospora* in the Sordariales is not without inconsistencies either. This order is characterized by dark perithecia or cleistothecia with tough, leathery peridia. Asci are produced in persistent, basal fascicles and usually have a thin refractive apical ring, although ascus walls may be evanescent in some species. The Sordariales also have both deliquescent paraphyses and pseudoparenchyma. Spores are variable in color, shape, and ornamentation, and many species have germ pores. *Melanospora* and its allies have a number of like characteristics, such as evanescent asci and variation in spore morphology and ornamentation, but still defy placement comfortably within this order.

Very little information exists concerning the biology and natural occurrences of this group of fungi. In addition, extensive confusion has prevailed over the years regarding the taxonomic placement of various genera within this ascomycete complex.

Therefore, this study was conducted in order to correctly identify and characterize a number of *Melanospora*-like pyrenomycetes isolated from production agriculture soils in Texas and Florida. A secondary goal was to identify and reclassify isolates of similar fungi that have been deposited in the University of Florida fungal herbarium.

Materials and Methods

Isolation

During the fall of 1994, while surveying Texas sugar beet-producing counties for isolates of the fusarial root rot pathogen, *Fusarium oxysporum* f. sp. *betae*., approximately 10-15% of cultures became contaminated after two-three weeks with an unknown pyrenomycetous ascomycete. Growth and vigor of the *Fusarium* isolates was severely affected in vitro with the dual cultures. The consistent association with and observed damage to the pathogen generated the concept of mycoparasitism and the utilization of this ascomycete as a potential biological control agent for fusarial wilt. Several cultures containing both organisms were dried and saved on sterile filter paper as previously described, and stored at 4°C (Harveson and Rush 1997).

Over the last 14 months, an additional five isolates closely resembling the Texas sugar beet *Melanospora* were obtained from agricultural fields in Florida. All were observed growing in dual culture with various *formae speciales* of *Fusarium oxysporum* recovered from diseased plant roots. These isolates reacted like the one from Texas by not becoming evident until after a period of several week's incubation. The recovered isolates were given a two letter acronym and are illustrated in Table 2-1 along with their provenance. The Texas sugar beet isolate was designated as TX, while the Florida isolates were named FL, FP, LE, GI, and GI-2. FL was found in association with the

causal agent of Fusarium wilt of Canary Island date palm, *F. oxysporum* f. sp. *canariensis*. Isolate FP was recovered from the tomato wilt pathogen, *F. oxysporum* f. sp. *lycopersici*, while the remaining three were isolated from the watermelon wilt pathogen, *F. oxysporum* f. sp. *niveum* (Table 2-1).

All isolates were separated from their respective hosts by one of two methods. Individual ascospore cirrhi were picked off culture plates and streaked on potato dextrose agar (PDA) or Mycological agar (MA). However, because of the difficulty of avoiding the numerous *Fusarium* microconidia, this was not always successfully accomplished. The other method entailed removing mature perithecia and shaking in two ml water blanks amended with 50% lactic acid. After several minutes, the water was spread on PDA and left at room temperature overnight. The following day, individual ascospores were removed with the aid of a dissecting microscope and transferred to PDA or MA amended with 100 ml/l of a filtrate from a three to four day old liquid culture of *Fusarium*. This greatly enhanced the germination and growth of the ascomycetes (Chapter 3).

Morphological Characterization and Identification

Cultures produced from germinated ascospores were transferred to and observed on 12 different growth media. Results of growth characteristics on these various media and subsequent inferred nutritional requirements are included in Chapter 3. The identification and correct taxonomic placement of these fungi was based upon current protocol for the Ceratostomataceae by Cannon and Hawksworth (1982), and Horie et al. (1986). This was accomplished by viewing and measuring the fungal morphological

Table 2-1. Isolates collected and used for taxonomic identification

Isolate Designation	Host ^a	Geographic Origin	Taxonomic Identity
FL	FOCA	Brevard Co., FL	<i>Sphaerodes retispora</i> var. <i>retispora</i>
FP	FOL	Indian River Co., FL	<i>Sphaerodes retispora</i> var. <i>retispora</i>
GI	FON	Gilchrist Co., FL	<i>Persiciasporea moreau</i>
GI-2	FON	Gilchrist Co., FL	<i>Sphaerodes retispora</i> var. <i>retispora</i>
LE	FON	Lake Co., FL	<i>Sphaerodes retispora</i> var. <i>retispora</i>
TX	FOB	Randall Co., TX	<i>Melanaspora zamiae</i>

^a FOCA = *F. oxysporum* f. sp. *canariensis*; FOL = *F. oxysporum* f. sp. *lycopersici*; FON = *F. oxysporum* f. sp. *niveum*; and FOB = *F. oxysporum* f. sp. *betae*

structures with both light and scanning electron microscopy. The shape, ornamentation, and germ pore structure were central criteria for establishing the identity of these fungi.

In addition to the six isolates found in association with *F. oxysporum*, another collection of isolates identified as *Melanospora* and deposited in the University of Florida fungal herbarium were examined and reclassified according to modern taxonomic criteria. These fungi included three isolates originally named as *Melanospora sphaerophila*, *M. ornata*, and *M. cameronse*. The *M. sphaerophila* isolate was collected originally in Kittery Point, ME in 1906 in association with *Plowrightia morbosa* on branches of *Prunus virginiana* (chokecherry). The *M. ornata* isolate was found in deer dung from Cheboygan Co., Michigan in 1969, while that of *M. cameronse* was found in Gainesville, Florida colonizing dead asparagus fern stems (*Asparagus plumosus*) in 1927.

Results

On PDA, all isolates except GI produced hyaline to white mycelium. Colony diameters of FL, FP, LE, and GI-2 reached 80 mm after six-seven days. GI grew at the same rate, but its mycelium was a creamy tan to straw color. Isolate TX was much slower-growing, attaining growth approximately one half that of the others in the same period of time. Mycelium on the agar surface was thick (4-8 μm) and verruculose, containing numerous bulbils (Fig. 2-1). These were particularly evident with increasing age of cultures. Aerial mycelium was much finer in structure measuring 0.5-2.0 μm in diameter with phialids scattered profusely along hyphal branches (Figs. 2-2 and 2-3). Most of the perithecia appeared to form within the branches of the aerial mycelium and not as readily on the agar surface.

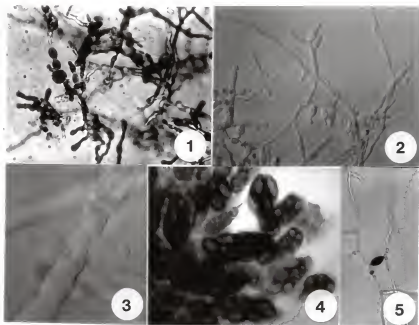
Hyphal growth on hemp agar was quite different than that on PDA. Aerial mycelium was sparse, and closely appressed to substrate surface. Mycelial growth occurred in an irregular, non-radial pattern, and perithecia were not as abundantly found as on PDA due to the lack of extensive aerial mycelium.

Although all isolates were approximately the same size and possessed the same morphological structures, several differences were also noted. FP was much more prolific in the production of this phialidic anamorph than the others. TX had the least amount of phialids, while the remaining four fungi were intermediate. In general, the production of perithecia likewise, differed greatly among isolates. Isolates LE and FL tended to form greater numbers of perithecia, while TX and GI produced the fewest. FP and GI-2 were intermediate between these two extremes. GI additionally formed its perithecia more often gregariously while the remaining taxa produced perithecia scattered more or less evenly over the substrate.

Perithecia were superficial, globose to ampullaceous, and amber to yellowish-brown in color. They formed fleshy, membranous walls composed of angular pseudoparenchymatous cells. All taxa had clavate, eight-spored asci that soon became deliquescent (Fig.2-4). Ascospores filled the perithecial cavity before being exuded out in a slimy cirrhous. Ascospores produced by all taxa were dark brown to olivaceous in color and ellipsoid to citriform in shape with two apical germ pores (Fig. 2-5). The light, translucent perithecia appeared dark to the naked eye due to the spore mass inside. All isolates were ostiolate with perithecial necks varying from 80-150 μm (Figs.2-6-2-11). The presence and lengths of coronal setae varied depending on growth substrate.

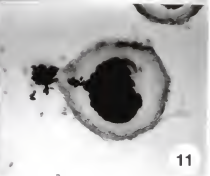
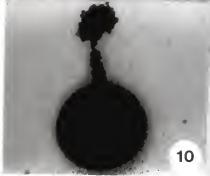
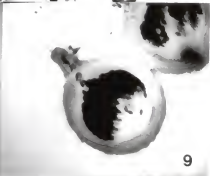
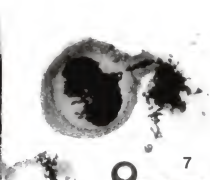
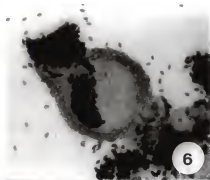
Figures 2-1-2-5. Morphological structures of *Melanospora* and allies

- 2-1. Hyphae at agar surface showing numerous bulbils (x 350)
- 2-2. Aerial hyphae showing anamorphic conidia or spermatia (x 350)
- 2-3. Close-up view of anamorphic state (x 500)
- 2-4. Squash mount of perithecium depicting asci in various states of maturity (x 500)
- 2-5. Germinating ascospore showing two polar germ pores (x 350)



Figures 2-6-2-11. Perithecia of *Melanospora* and allies. Note that all are ostiolate.

- 2-6. *Sphaerodes retispora* var. *retispora* (isolate FL) (x 138)
- 2-7. *Sphaerodes retispora* var. *retispora* (isolate FP) (x 123)
- 2-8. *Sphaerodes retispora* var. *retispora* (isolate GI-2) (x 127)
- 2-9. *Sphaerodes retispora* var. *retispora* (isolate LE) (x 126).
- 2-10. *Melanospira zamiae* (isolate TX) (x 150)
- 2-11. *Persiciospora moreau* (isolate GI) (x 141)



However, despite numerous similarities among isolates, there were some major differences that are commonly used now to separate members of this group of fungi. These include the structure of the germ pore and the morphology and ornamentation of the ascospore. Spores of isolates LE, FL, FP, and GI-2 were all coarsely reticulate with strongly apiculate germ pores. They contained 8-10 deep lumina on each face of the spore (Figs. 2-12-2-15). The spores of TX were smooth with sunken or depressed germ pores (Fig. 2-16). GI produced spores that were also smooth in outline, but differed from the others by having finely pitted spore walls reminiscent of a peach seed, with slightly apiculate germ pores (Fig. 2-17). A comparison of morphological structures and sizes among isolates is presented in Table 2-2.

Based upon all discussed taxonomic criteria, isolates LE, FL, FP, and GI-2 were concluded to be *Sphaerodes retispora* var. *retispora*. TX was classified as the type species for *Melanospora*, *M. zamiae*. GI was determined to be the third reported finding for the newly created taxon, *Persiciospora moreauli* (Cannon and Hawksworth 1982) (Table 2-1).

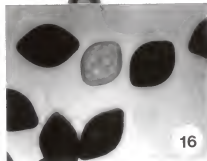
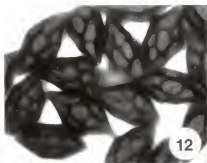
One of the three herbarium specimens was not able to be identified to species. *Melanospora cameronse* was correctly named at the generic level due to its dark, smooth ascospores with depressed germ pores (Fig. 2-18). However, the sample was in poor shape and perithecia were seldom able to be observed. They disintegrated upon contact with the cover slip and all that remained was a mass of spores in the place previously occupied by the perithecium (Fig. 2-19). Since no immature perithecia could be located, no asci could be examined. An extensive search of the literature did not reveal other

Table 2-2. A comparison of biological and morphological characteristics among *Melanospora* and its allies.

Specimen	Genus	Geographic Origin	Perithecium (μm)	Ascus (μm)	Ascospore (μm)	Ostiole	Substrate	Spore Morphology
FL	<i>Sphaerodes</i>	Florida	240-320	35-50 x 18-22	18-22 x 8-12	Yes	<i>F. axysporum</i>	Reticulated
FP	<i>Sphaerodes</i>	Florida	280-320	35-55 x 18-22	20-22 x 8-12	Yes	<i>F. axysporum</i>	Reticulated
GI	<i>Persiciasporea</i>	Florida	250-300	40-45 x 20-22	20-22 x 8-10	Yes	<i>F. axysporum</i>	Pitted
GI-2	<i>Sphaerodes</i>	Florida	240-300	35-50 x 18-21	18-20 x 8-10	Yes	<i>F. axysporum</i>	Reticulated
LE	<i>Sphaerodes</i>	Florida	240-350	35-50 x 18-22	18-22 x 10-12	Yes	<i>F. axysporum</i>	Reticulated
TX	<i>Melanospora</i>	Texas	230-300	40-65 x 20-25	16-18 x 12-14	Yes	<i>F. axysporum</i>	Smooth
<i>M. sphacaphila</i>	<i>Scopiniella</i>	Maine	250-375			Yes	<i>Prunus virginiana</i>	Smooth
<i>M. annata</i>	<i>Sphaerodes</i>	Michigan	220-400			Yes	Deer dung	Reticulated
<i>M. camerense</i>	<i>Melanospora</i>	Florida	?			?	<i>Asparagus plumosus</i>	Smooth

Figures 2-12-2-17. Mature ascospores for *Melanospora* and allies.

- 2-12. *Sphaerodes retispora* var. *retispora* (FL), note the reticulations with 8-10 lumina on the face of each spore. (x 1500)
- 2-13. *Sphaerodes retispora* var. *retispora* (FP), note same structures. (x 1476)
- 2-14. *Sphaerodes retispora* var. *retispora* (GI-2), note same structures (x 1578)
- 2-15. *Sphaerodes retispora* var. *retispora* (LE), note same structures. (x 1550)
- 2-16. *Melanospora zamiae* (TX), note smooth spores with no additional ornamentation. Lighter spore is immature. (x 1470)
- 2-17. *Persiciospora moreau* (GI), note pores or pitted surface of spore characteristic of this genus. (x 1309)

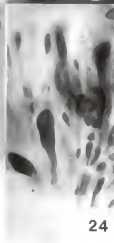


reports of similar fungi containing the specific epithet, *cameronse*. Therefore, the name used for this specimen, *M. cameronse*, was retained. Conversely, the remaining two specimens were easily identified and redesignated with their currently accepted generic names. The specimen labeled as *Melanospora ornata* needed to be renamed. This taxon had been synonymized with *Sphaerodes fimicola* (Cannon and Hawksworth 1982). The sample easily fit into this category because of its eight-spored asci (Fig. 2-20), globose perithecium (220–400 μm), very short neck and poorly developed coronal setae, and dark, citriform spores with strongly apiculate germ pores and coarse reticulations (Fig. 2-21).

The other specimen from the herbarium that needed renaming was *Melanospora sphaerophila*. It had been synonymized with and described as *Scopinella sphaerophila* by Malloch in 1976. The herbarium sample proved to be identical with Malloch's description (1976) by possessing black, carbonaceous perithecia measuring 250–375 μm in diameter with very long necks (450–700 μm) (Fig. 2-22). Its smooth, barrel-shaped ascospores contained a thick walled, dark brown band in the middle of the spore with hyaline, protruding walls at each end that collapsed bilaterally after drying (Malloch 1976) (Fig. 2-23). This feature was thought to be a vertical germ slit by Cannon and Hawksworth (1982), but *Scopinella* was later proven to germinate from both lateral, hyaline protuberances (Tsuneda et al. 1985). Its identification was further confirmed by the presence of the unique, 2-spored asci (Malloch 1976, Tsuneda et al. 1985) (Figs. 2-24 and 2-25).

Figures 2-18-1-25. Morphological characteristics from specimens deposited in University of Florida fungal herbarium.

- 2-18. Mature ascospore of *Melanospora cameronse*. Note the smooth spore with no ornamentation (x 1500)
- 2-19. Ascospores of *M. cameronse* after mounting. Clump of spores are the only structures remaining after degradation of perithecium (x 500)
- 2-20. Eight-spored, clavate ascus of *Sphaerodes fimicola* (x 404)
- 2-21. Mature ascospores of *S. fimicola*. Note the fine reticulations on spore surface (x 1300)
- 2-22. Perithecium of *Scopinella sphaerophila*, note the long neck (x 160)
- 2-23. Mature ascospores of *S. sphaerophila* (x 1375)
- 2-24. Immature asci of *S. sphaerophila* arising from an ascogenous hyphal system (x 1100)
- 2-25. Nearly mature ascus of *S. sphaerophila*. Note characteristic two-spored nature (x 1535)



Discussion

The difficulty in classifying allied members of the Ceratostomataceae has been well documented. Horie et al. (1986) state that the reason for this fact is that superficially similar ascomycetes that are placed in this group have all been derived from other unrelated sources through convergent evolution. For example, the presence of the deliquescent ascus, long necks, and ostiolar setae are adaptations for passive dispersal of spores by animal movement or rain splashing, rather than active dispersal by air currents via forcibly discharged ascospores and poroid asci. Horie et al. (1986) further point out that the characters now utilized for taxonomic separation, such as ascospore morphology and ornamentation, and germ tube structure, are useful as indicators of true evolutionary relationships since they would not be subjected to strong selection pressures.

Recently, the tremendous insurgence of analyses using molecular techniques and characters and the resulting inferred phylogenies among pyrenomycetes has not extensively addressed this group of fungi. Very few members of this group consisting of *Melanospora* and its closely related allied genera have been included in these types of studies. However, two recent ones have been attempted utilizing *M. zamiae*, and *M. fallax* (Rehner and Samuels 1995, Spatafora and Blackwell 1994). In both cases, *Melanospora* was derived within or near the Hypocreales, specifically in clades containing the genus *Nectria*. Although *Melanospora* and its allies were once included in this order by some workers, they have many distinctly non-Hypocrealean features. They are similar to the Hypocreales in the enteroblastic, phialidic anamorphs, and the fleshy, light colored, true perithecia. However they differ substantially from hypocrealean fungi by containing deliquescent asci, and dark, aseptate ascospores with polar germ pores.

In a separate study, inference with another group of fungi has been found. These same two *Melanospora* species, *M. zamiae* and *M. fallax* have additionally been aligned phylogenetically with the anamorphic, ambrosial species *Raffaella hennebertii* (Jones and Blackwell 1998). Members of this genus have been difficult to derive because of the lack of a known teleomorphic state. However, when *R. hennebertii* was excluded from the analysis, seven different species from this genus formed a lineage that was resolved as a sister group with the sexual genus *Ophiostoma* (Jones and Blackwell 1998). These modern taxonomic studies have therefore proven to be equally as difficult to discern and understand as have the morphological ones.

As pointed out earlier, the primary reasons for the problems of taxonomic placement for this group of pyrenomycetes is shared characteristics, both morphological and molecular, with other fungal taxa. This can be further illustrated with the example of *Sordaria destruens*. This taxon was first referred to *Anthostomella* before being transferred to *Melanospora* by Shear (Shear and Dodge 1927). It was used for almost thirty years as the model for studying fungal physiology and nutrition before being transferred to *Sordaria* in 1951 (Asthana and Hawker 1936, Hawker 1936, 1951). The inclusion of this taxon in the literature for many years as *Melanospora* has likely contributed to the confusion between this genus and its allied genera in subsequent studies, as well as contributing to the incorrect placement of these species.

CHAPTER 3

ENHANCEMENT OF GROWTH AND SPORULATION OF *MELANOSPORA* AND ITS ALLIES BY *FUSARIUM OXYSPORUM*

Introduction

The phenomenon of enhanced sporulation of fungi by other organisms has been observed for many years. The first account of increased reproduction of one organism by another was recorded by Molliard in 1903. He found that species of *Ascobolus* growing on carrots produced numerous apothecia in the presence of a contaminating bacterium, but not when growing in pure culture (Molliard 1903). The first report of the enhancement of sporulation by one fungus on another was made by Heald and Poole (1909) in Nebraska. They found that *Melanospora pampeana* (syn. *M. zamiae*) produced perithecia abundantly in a mixed culture with *Fusarium moniliforme*, but only gave sparse mycelial growth after being in pure culture (Heald and Poole 1909). A recent paper by Watanabe (1997) has also demonstrated a similar process for *Sordaria fimicola* and two species of *Armillaria*. In this case, *A. mellea* and *A. tabescens* were antagonistic to *S. fimicola* by inhibiting its growth. However, at the same time, these *Armillaria* species increased perithecium and ascospore production of *Sordaria*.

Many different fungi have also been demonstrated over the years to need certain nutrients for adequate growth and sporulation in pure culture. A growth substance found to be important for maximum culture of certain yeasts was identified in the late 1920's. Eastcott (1928) was able to separate this substance into two parts, one of which was

identified as inositol. This compound is now known to be a hexahydroxycyclohexane that occurs widely in the phospholipids of many microorganisms (Singleton and Sainsbury 1987). Robbins and Kavanagh (1938) showed that the addition of vitamin B₁ (thiamin) promoted the growth of *Phycomyces blakesleeanus* in synthetic culture. Using an isolate of *Melanospora destruens* (syn. *Sordaria destruens*), Hawker (1938,1939) found that its growth on a medium containing glucose and various inorganic salts was negligible. The addition of thiamin had no beneficial growth effect on the fungus. Biotin (vitamin K) added to the same medium, gave good vegetative growth, but no perithecial production. However, when both biotin and thiamin were included, both reproduction and mycelial growth were vastly improved.

Jordan and Barnett (1978) were similarly able to induce sporulation of *M. zamiae* better in dual culture with other fungi than by itself. They additionally attained greater sporulation and hyphal growth with biotin and thiamin added to a glucose-based medium, but discovered that the addition of zinc enhanced these properties to an even greater extent. No growth of the fungus occurred without the inclusion of these nutrient supplements. (Jordan and Barnett 1978). Conversely, Vakili (1989) found that the majority of *Melanospora dammosa* isolates produced from single ascospores would germinate on PDA. His results determined that only 6% and 2% of the isolates required thiamin and biotin respectively for germination and axenic growth.

A number of fungi have also been observed occurring and growing in close association with other fungi. These types of relationships have also been noticed to be necessary for the growth and sporulation of some members. Those members of these complexes presumed to be parasitic were often not successfully grown in pure culture

(Jordan and Barnett 1978, Whaley and Barnett 1963). *Gonatobotrys simplex* was found in association with *Alternaria tenuis* and attempts to grow it axenically failed unless an extract of the *Alternaria* was added to the medium (Whaley and Barnett 1963). A growth factor was isolated and partially purified from this extract and termed mycotrophein. It was assumed to be vitamin-like because it was only needed in minute amounts (Whaley and Barnett 1963), but its nature is still not fully understood.

Gonatobotrys and several species of *Melanospora* have also been demonstrated to be fusion biotrophs of other fungi (Jordan and Barnett 1978, Jeffries and Young 1994, Vakili 1989). The biological behavior of these types of parasites appears to often be associated with a nutritional dependency (Jeffries and Young 1994). It is assumed that whatever growth factor or vitamin-like molecule that is responsible for enhanced growth and sporulation of these organisms in culture can be obtained directly from the host hyphae in nature.

The collection of pyrenomycetes found naturally occurring in association with *F. oxysporum*-infected plant roots (Chapter 2) may also be reacting in a similar manner. Species of *Sphaerodes* and *Persiciospora*, like *Melanospora*, have been suspected as being parasitic on other fungi (Cannon and Hawksworth 1982). It is not known, however if any of them exhibited a similar dependency upon their hosts or host products for improved sporulation and growth. Therefore, this study was initiated to explore this possibility and to better characterize the association and interrelationships between members of this fungal group and the various *formae speciales* of *F. oxysporum* from which they were originally found.

Materials and Methods

Since all pyrenomycete isolates were found occurring in dual culture with *F. oxysporum*, it was first necessary to separate the two organisms and isolate them in pure culture. Once separation had been accomplished, it was quickly observed how slow the pyrenomycetes germinated and grew on a selected group of standard synthetic media.

Preliminary attempts to grow the isolates using different concentrations of a *Fusarium* culture filtrate provided promise for increased sporulation and growth. These experiments consisted of filtrates from four-five day old *F. oxysporum* cultures grown in a liquid medium composed of sucrose, yeast extract, and inorganic salts (Esposito and Fletcher 1961). Cultures were vacuum-filtered through 150 ml bottle top flasks (0.45 μ m capacity, Corning, Corning, NY) directly into media bottles using a hand-held Nalgene (Cucamonga, CA) vacuum pump. Concentrations of the filtrate consisted of 25, 50, and 100 ml per one half liter of media. These experiments also included the addition of filtrate before and after autoclaving. Filtrates produced by the still culture from the four *formae speciales* of *F. oxysporum*, *niveum*, *lycopersici*, *canariensis*, and *betae*, were all evaluated on PDA and mycological agar with the corresponding pyrenomycetes in all possible combinations. Once methods and techniques for optimum efficiency were established, all subsequent tests routinely included filtrate from *F. oxysporum* f. sp. *niveum* (FON) at a rate of 50 ml per one half liter of media added prior to autoclaving.

Effects of Media with and without the Addition of Filtrate

Isolates used for this study included the four *Sphaerodes* strains and the one isolate of *Melanospora* (Chapter 2). *Persiciospora moreau* was include in the preliminary

studies, but was eventually lost in culture and was never able to be reconstituted. It has, however, been deposited in the University of Florida fungal herbarium and is available for microscopic examination.

There were 12 different media employed for this portion of the study. They and their ingredients are illustrated in Table 3-1. These media were used in to determine the most efficient source for growth and reproduction of these fungi for eventual mass production of inoculum. It was also hoped that inferences could be made on the nutritional requirements or preferences for this group of pyrenomycetes.

An eight mm diameter cork borer agar plug was placed in the center of each plate utilizing six replications per medium. Each medium was evaluated both with the added filtrate (50 ml/1/2 liter) and without. Plates were incubated in the dark at 25°C. Colony growth measurements were made after six days. After measurements were recorded, plates were further incubated for another two weeks.

After the two week incubation period, growth morphology and characteristics were recorded. Four cork borer plugs (eight mm) were also removed approximately one inch away from the point of plate inoculation at the 12, 3, 6, and 9 o'clock positions. Each plug was viewed under a dissecting microscope and perithecia were counted. Four of the 24 plugs utilized for counting perithecia were randomly selected, prepared as squash mounts, and observed microscopically. With the use of 10X magnification, 12 perithecia from each plug were randomly measured for diameter at the widest point.

Table 3-1. Media used for evaluation of growth, perithecial development, and perithecial size for isolates of *Melanospora zamiae*, and *Sphaerodes retispora* var. *retispora*.

Medium	Ingredients
AK sporulating agar (AK)	Pancreatic digest of gelatin, pancreatic digest of casein, yeast extract, beef extract, dextrose, and $MgSO_4$
Brain infusion agar (BIA)	Infusion of calf brains and beef hearts, proteose peptone, dextrose, NaCl, Na_2PO_4
Cornmeal agar (CMA)	Infusion of cornmeal
Emerson's YPS (EYPS)	Yeast extract, soluble starch, K_2PO_4 , $MgSO_4$
Hemp agar (HA)	Sterilized hemp seeds
J&B agar	glucose (dextrose), casein hydrolysate, $MgSO_4$, thiamin, biotin, microelement soln - (Mn, Zn, Fe)
Komada's agar (KA)	galactose, asparagine, K_2PO_4 , KCl, $MgSO_4$, Fe-Na EDTA, NaB_4O_7 , PCNB, oxgall powder, streptomycin SO_4
Malt extract agar (ME)	maltose, dextrin, glycerol, peptone
Mycological agar (MA)	soytone, dextrose
Potato dextrose agar (PDA)	Infusion of potatoes, dextrose
Starch agar (SA)	Soluble starch, beef extract
V8 ² (double strength)	V8 juice, CaCO

Factorial Study

This study was initiated to more fully explore and understand possible interrelationships between each of the four *Fusarium formae speciales* and their corresponding fungal partner with which they were recovered. There were two primary questions which were being addressed. One was whether there was an added benefit with the inclusion of a filtrate above that achieved with the host *Fusarium* alone. The second was whether evidence could be gathered that suggested if a host specificity or preference was involved with each *forma specialis* and the specific fungus found with it. The major assumption being made with this approach was that the ability of the each fungus to reproduce in conjunction with the *Fusarium* was an important factor in host-parasite evaluations. Therefore, perithecial production was the central criterion that was used for evaluating this process. Peripheral goals that could be achieved at the same time included evaluation of medium effect, relative efficiency of parasites to sporulate, and efficiency of *formae speciales* to induce formation of perithecia.

This study was designed as a four-level factorial using four media, four *formae speciales*, four suspected parasites, and the inclusion or exclusion of filtrate in media (Table 3-2). Each host and parasite were paired on each medium one half inch apart in all possible combinations using five replications for each combination. Each medium was also evaluated with filtrate added or excluded. After two week's incubation in the dark, plugs were removed along two transect lines on each plate, four plugs spaced equidistantly from each transect (Fig. 3-1). Perithecia were counted on the eight plugs as before with the aid of a dissecting microscope.

Table 3-2. Media, pyrenomycete isolates and *Fusarium oxysporum* isolates utilized in the factorial study.

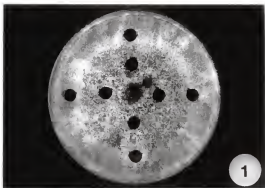
Medium	<i>F. oxysporum</i> forma <i>specialis</i>	Parasite
Potato dextrose agar	<i>betae</i>	TX
Double strength V8 agar	<i>canariensis</i>	FL
Emerson's YPS agar	<i>lycopersici</i>	FP
Starch agar	<i>niveum</i>	LE

TX = *M. zambiae*

FL, FP, and LE = *S. retispora* var. *retispora*

Each host-parasite combination was evaluated on each medium in all possible combinations, and each medium was tested both with and without the addition of a filtrate.

Figure 3-1. Plate inoculated with both *F. oxysporum* and pyrenomycete for evaluation of perithecial development in the factorial study. Note the plugs removed along two transect lines. Perithecial counts were made from each plug. Tiny black structures are mature perithecia (x 0.8)



Both studies were repeated once, and all data were analyzed by the general linear model procedure. The means involving growth, sporulation, and perithecial size in the presence or absence of filtrate on different media were separated by Duncan's mean separation test. In the factorial study, all possible interactions and main effects were tested before separating means by the least squares procedure (LS Means). The pairwise probability values obtained were additionally adjusted by the Bonferroni correction.

Filtrate Evaluation

Finally, two separate assays were attempted to establish some metabolic or biological properties of the filtrate produced by the *Fusarium* isolates. Both involved the use of dialysis tubing with a cut-off size of 14-16,00 MW (Spectra/Por, Spectrum Medical Industries, Los Angeles, CA). Strips of tubing were taped to pieces of wire mesh and autoclaved. These obstructions were placed in the center of Petri plates, dividing them in half. Plugs of both parasite and *Fusarium* were inoculated on separate sides with the parasite alone on separate plates as controls. Colony diameters of the parasite from both treatments were measured after one week.

The other assay consisted of filling a six inch piece of tubing with 15 mls of PDA broth and two plugs of the *Fusarium*. The ends were sealed and placed into sterile media bottles containing 500 mls of PDA broth and two plugs of the parasite. This was compared to bottles of liquid media containing only the parasite. After 10-14 days, dialysis tubing was removed, contents were strained through cheesecloth and weighed.

Results

After separation of isolates of the *Melanospora* and *Sphaerodes* from the *F. oxysporum*, all isolates were evaluated after growing from a single ascospore. Because they all grew and sporulated to some extent on the different media, they were concluded to be homothallic and also not obligate parasites of the various *formae speciales*. From the preliminary experiments, it was further determined that no differences existed in terms of growth or perithecial production in response to the filtrates produced by the *Fusarium* isolates. Because watermelon wilt was the pathosystem to be eventually evaluated for disease control, filtrate from the *Fusarium niveum forma specialis* was routinely used for all subsequent studies. It was also observed that few differences existed with the volume of filtrate added to each medium. Therefore, a 1:10 ratio of filtrate to medium volume was selected for all subsequent evaluations and general maintenance of isolates (50 ml/1/2 liter).

Linear growth after six days was significantly enhanced for all isolates, as was perithecial production (Tables 3-3 and 3-4). However, the addition of filtrate had no effect upon perithecial diameter (Tables 3-3 and 3-4). Significant differences were observed among the three variables, and were influenced by choice of medium and pyrenomycete isolate. These differences among growth, perithecial production and size are illustrated in Tables 3-5 and 3-6 for combined media and isolates respectively.

With the exception of J&B, the better media for growth tended to be better for sporulation as well. This medium consisted of glucose and inorganic salts amended with biotin, thiamin, and a micronutrient solution (Mn, Zn, Fe), and was developed exclusively for a *M. zamiae* from West Virginia (Jordan and Barnett 1978). However, the *M. zamiae*

isolate used in this study from Texas did not respond well to this medium. In terms of sporulation, none of the other *Sphaerodes* isolates in this study responded well either (Table 3-5).

Although among the leaders at producing linear growth, J&B medium was extremely poor in inducing perithecial production (Table 3-5). The media that overall allowed the best combination of growth and sporulation were PDA, MA, AK, and ME (Table 3-5). These media also tended to produce the greatest amount of fluffy, aerial mycelium, which is where the majority of the perithecia were formed. Those media that did not induce overall good, vigorous growth and perithecial formation included Komada, starch, Emerson, hemp, and cornmeal agars. Brain infusion agar was similar to J&B by producing extensive mycelial growth, but the numerous perithecia produced after two weeks were primarily immature. Interestingly, the Komada medium allowed absolutely no growth at all for any of the pyrenomycete isolates. This is a *F. oxysporum*-selective medium that truly worked correctly by inhibition of the pyrenomycetes completely. It was determined that a plug of any of these isolates could be placed on this medium for at least a month without producing any growth. However, the isolates were not killed, because the plugs could be removed from the Komada, placed on a suitable growth medium and afterward function normally.

Those media having high soluble starch (starch, Emerson) or cellulose content (V8², hemp, cornmeal) produced very little aerial mycelium. Growth was often extensive linearly but very thin, sparse and flat to the agar surface. Perithecial development was additionally poor (Table 3-5). In general, perithecial diameter was

Table 3-3. General linear model data for growth, perithecial production, and perithecial diameter of pyrenomyces isolates with and without an added filtrate from *F. oxysporum* f. sp. *niveum*.

Source	df	growth (mm)	Pr > F	
			perithecia	perithecial size (μm)
Form	1	0.0001	0.0001	0.8743*
Medium	11	0.0001	0.0001	0.0001
Isolate	4	0.0001	0.0001	0.0001
Replication	5	0.9254*	0.9481*	0.3830*

* = not significant at $P = 0.01$

Table 3-4. The effect of an added fusarial filtrate to growth, perithecial production and perithecial diameter of pyrenomyces isolates.

	Growth (mm)	Perithecia	Perithecial size (μm)
Filtrate	68.3 a	26.4 a	245.6 a
No Filtrate	38.9 b	10.6 b	245.7 a

Means followed by the same letter are not significantly different according to Duncan's multiple range test (P =0.01)

Table 3-5. Growth, perithecial production and perithecial size of all pyrenomyces isolates combined on different growth media.

Medium	Growth (mm) ^a	Perithecia ^a	Perithecial size (μm) ^a
AK	49.0d	29.9c	224.8ef
brain infusion	56.7c	4.9f	220.8f
cornmeal	30.5f	10.9e	256.8b
Emerson's YPS	50.2d	14.1e	277.6a
hemp	47.4d	12.2e	251.2bc
J&B	57.2c	3.7f	244.0cd
Komada's	0.0g	0.0h	0.0h
malt extract	58.9c	20.2d	199.2g
mycological	72.3b	38.4b	234.4de
potato dextrose	79.7a	42.2a	231.2ef
starch	47.5d	11.6e	277.6a
V82	38.9e	12.6e	273.6a

Means followed by the same letter are not significantly different according to Duncan's multiple range test.
(P = 0.01)

Table 3-6. Growth, perithecial production and perithecial diameter for each pyrenomycete isolate on all combined growth media.

Isolate ^a	Growth (mm) ^b	Perithecia ^b	Perithecial size (μm) ^b
FL	54.3b	28.3a	261.6a
FP	59.7a	20.6a	237.6b
GI-2	54.2b	20.5a	232.0b
LE	59.6a	22.8a	259.2a
TX	39.9c	5.7b	233.6b

^aFL, FP, GI-2, and LE = *Sphaerodes retispora* var. *retispora*; TX = *Melanospora zamiae*

^bMeans followed by the same letter are not significantly different according to Duncan's multiple range test.

(P = 0.01)

likewise influenced by choice of growth media and isolate. Those that produced the poorest growth and sporulation also tended to induce significantly larger perithecia (Table 3-5). Isolates FL and LE were observed to form larger, but not more numerous perithecia than the other pyrenomyces with the exception of the *M. zamiae* isolate from Texas (Table 3-6).

Because of the significant differences detected among media and isolates, means of the measured variables were sorted by fusarial filtrate effect and evaluated individually. Tables 3-7 and 3-8 depict the effects of filtrate and medium upon the growth, and sporulation, respectively, of the various isolates being evaluated in this study. Isolate TX (*M. zamiae*) was consistently the slowest-growing and produced the fewest perithecia among the other *Sphaerodes* isolates evaluated. This is particularly evident in Table 3-8 showing its production of perithecia without filtrate. In general, most media with the added filtrate greatly improved both sporulation and growth, but ME was an exception. With sporulation of isolates FL and LE, the difference between treatments was minimal. Conversely, isolate FP actually produced more perithecia with the non-filtrate treatment (Table 3-8). The linear growth produced by these isolates on PDA and mycological agars could also be misleading. Although very little difference is noticed from looking at the two treatments in Table 3-7, the filtrate was still better in inducing luxuriant, vigorous aerial growth than was the same media without the filtrate. A more accurate picture of the beneficial effect of filtrate is observed with the production of perithecia (Table 3-8)

The factorial study provided further evidence of the importance or dependence of these isolates upon *F. oxysporum*. After analyzing the perithecial production from the different pyrenomyces paired with the four *formae speciales*, it was determined that the

addition of filtrate to media is not a significant factor, like it had been with the pyrenomycetes growing by themselves. The general linear model data obtained for main effects and all possible interactions is presented in Table 3-9. Because all main effects except for filtrate were significant, means from different treatments were separated and are included in Table 3-10. After combining results for all fungal isolates, PDA was shown to produce the greatest number of perithecia followed by V82, starch and Emerson. Isolates LE and FL formed greater numbers of perithecia on all media and *formae speciales* followed by FP and TX. Finally, the sugar beet pathogen (FOB) was the poorest host for all pyrenomycetes and media, whereas no differences were observed among the remaining *formae speciales* (Table 3-10).

The major questions being asked about the relationships between the pyrenomycetes and the *Fusarium formae speciales* were effectively answered by this complicated factorial study. There were no significant differences observed with the filtrate treatment, or with any interaction that included filtrate (Table 3-9). This essentially answered the first question. Whatever advantage the pyrenomycetes received from the filtrate was likewise obtained from the *Fusarium* isolates themselves, and there was no added benefit to the filtrate in the media. However, there was a significant interaction involving the pyrenomycete-*F. oxysporum* combination (Table 3-9), which alluded to the second question being asked from these organismal relationships. Therefore, it was important to investigate these relationships more completely with an additional analysis.

Table 3-11 shows the results of this additional analysis after subjecting data to the least square means and pairwise comparison procedures. Isolate LE was the only one of

Table 3-7. Linear growth (mm) among pyrenomycete isolates after six day's incubation as influenced by media and addition or exclusion of fusarial filtrate.

Medium	Filtrate					No Filtrate				
	Isolate ^{a,b}									
	FL	FP	GI-2	LE	TX	FL	FP	GI-2	LE	TX
AK	49.3c	71.3a	60.3b	62.3b	47.3c	32.3c	45.0a	39.7b	46.7a	35.3c
Brain	63.7b	73.7a	54.0c	69.0ab	47.0d	50.3b	66.0a	43.7c	66.7a	33.0d
CM	48.3a	46.3a	46.3a	47.3a	47.0a	14.0a	13.7a	15.0a	13.7a	13.0a
Emerson	79.3b	85.0a	84.3a	85.0a	53.7c	21.0b	32.7a	32.7a	17.3c	10.7d
Hemp	70.0a	72.0a	71.7a	69.0ab	64.3b	29.0a	24.7b	24.7b	28.3a	22.7b
J&B	85.0a	85.0a	80.0a	85.0a	64.7b	30.7d	43.3b	35.3c	49.0a	14.0e
Komada	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
ME	76.7a	75.7a	72.0a	76.0a	55.7b	37.3c	54.0a	54.0a	48.0b	40.0c
Mycol.	85.0a	85.0a	85.0a	85.0a	69.0b	73.7b	74.3b	53.3c	79.3a	33.3d
PDA	85.0a	85.0a	85.0a	85.0a	85.0a	83.3a	83.0a	80.0a	84.0a	41.7b
Starch	68.3b	80.7a	81.0a	81.0a	49.7b	25.3b	22.7b	28.7a	24.0b	14.0c
V8 ²	58.3b	51.7c	34.0d	68.0a	26.0e	29.3d	45.0a	32.2c	42.3b	12.0e

^aFL, FP, GI-2, and LE = *Sphaerodes retispora* var. *retispora*; TX = *Melanospora zamiae*

^bMeans followed by the same letter are not significantly different according to Duncan's multiple range test.

(P = 0.01)

Table 3-8. Perithecial production among pyrenomycete isolates after two week's incubation as influenced by media and addition or exclusion of *Fusarium* filtrate.

Medium	Filtrate					No Filtrate				
	Isolate ^{a,b}									
	FL	FP	GI-2	LE	TX	FL	FP	GI-2	LE	TX
AK	44.7b	42.7b	37.1b	61.0a	33.8b	11.4c	26.5a	22.0ab	13.0bc	7.3c
Brain	20.2a	2.7bc	5.0b	18.5a	0.7c	0.9ab	0.7b	0.0c	1.3a	0.0c
CM	21.2b	28.1a	29.1a	19.7b	11.1c	0.0a	0.0a	0.0a	0.0a	0.0a
Emerson	20.5bc	29.3a	24.2ab	19.0bc	17.7c	6.7b	10.5a	7.0b	4.6b	1.1c
Hemp	21.1ab	24.3ab	28.2a	17.3ab	15.5b	1.5b	0.0c	0.0c	2.0a	0.0c
J&B	7.3a	9.5a	0.0b	8.5a	0.0b	0.0b	3.2a	5.0a	3.2a	0.0b
Komada	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a
ME	40.2a	7.4c	24.7b	26.3b	0.0c	33.6c	22.9b	0.0d	25.1b	1.2c
MA	66.6a	48.7b	28.2c	63.8a	15.1c	41.5a	24.0b	49.2c	46.7a	0.0d
PDA	77.4a	67.1a	84.1a	75.6a	1.7b	42.2b	51.7a	31.8c	42.6b	0.0d
Starch	22.4ab	23.5ab	27.9a	18.2b	17.5b	1.4a	2.3a	1.5a	1.3a	0.0b
V8 ²	40.0a	27.7a	5.1b	37.3a	2.4b	3.4a	4.5a	0.7b	4.7a	0.0c

^aFL, FP, GI-2, and LE = *Sphaerodes retispora* var. *retispora*; TX = *Melanospora zamiae*

^bMeans followed by the same letter are not significantly different according to Duncan's multiple range test. (P= 0.01)

Table 3-9. General linear model data describing sporulation of four pyrenomycetes paired with their four corresponding *F. oxysporum formae speciales*.

Source	df	Pr > F
Medium	3	0.0001
Pyrenomycete isolate	3	0.0001
Filtrate	1	0.8866*
<i>Fusarium</i> isolate	3	0.0001
Replication	4	0.8492*
Filtrate*Pyrenomycete isolate	3	0.2249*
<i>Fusarium</i> *Pyrenomycete isolate	9	0.0001
Filtrate* <i>Fusarium</i> isolate	3	0.2699*

* = not significant at $P = 0.01$

Table 3-10. Factorial study data illustrating perithecial development for pyrenomycete isolates, *Fusarium* isolates, and growth media.

Mean ^a	Medium	Pyrenomycete isolate ^b	<i>Fusarium</i> isolate ^c
63.2	PDA a		
57.9	V8 ² b		
43.4	Starch c		
36.9	Emerson d		
60.5		LE a	
50.3		FL b	
45.5		FP c	
45.2		TX c	
51.8			FOL a
51.7			FOCA a
50.3			FON a
47.6			FOB b

^aMeans followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P = 0.01$).

^bLE, FL, FP = *Sphaerodes retispora* var. *retispora*; TX = *Melanospora zamiae*

^cFOL = *F. oxysporum* f. sp. *lycopersici*; FOCA = *F. oxysporum* f. sp. *canariensis*; FON = *F. oxysporum* f. sp. *niveum*; and FOB = *F. oxysporum* f. sp. *betae*

the four pyrenomyces that was capable of producing a significantly greater number of perithecia on its original host from the field. In fact, no other isolate was able to match LE's sporulation efficiency on any of the *formae speciales*. This indicated that this isolate was better than the others at reproducing on this group of *Fusarium* isolates. Although numerous other examples of differences among and between isolates were detected, there were no further incidences of significant perithecial production associated with each pyrenomycete and the *forma specialis* from which it was obtained. So this effectively addressed the second question and proved that only the combination including isolate LE and *F. oxysporum* f. sp. *niveum* appeared to exhibit a significant host specificity or preference in terms of reproductive ability.

The experiments with the dialysis tubing provided little information on the chemical nature of the filtrate, however a great deal was learned about its biological properties. Whatever metabolite or growth factor that was being produced in response to the culture of *Fusarium* was heat and pressure stabile. It was able to retain its activity of enhancing growth and sporulation regardless of whether it was added to media before or after autoclaving. Secondly, it had a molecular weight smaller than 12-14,000, which was the cut-off size for the pores in the dialysis tubing. Both assays demonstrated that the active ingredient in the filtrate could pass through the tubing and still maintain its beneficial effect upon growth and perithecial production for the pyrenomyces.

Discussion

The reported nutritional requirements for *Melanospora* growth and sporulation have proven to be conflicting over the years. In many cases this study's results do not correlate

with those of others. For example, Hanlin et al. (1993) and Goh and Hanlin (1994), successfully utilized V8 juice agar for both culture maintenance and developmental studies because of superior perithecial production over that of ME agar. The *M. zambiae* isolate originally found in Texas was more effective in terms of growth and reproduction on ME than on V8. Perithecial production on V8 by isolate TX was among the poorest of all media evaluated, and also resulted in very slow, limited growth (Tables 3-5, 3-9, and 3-10). However, V8 was among the better media for growth and sporulation of several of the *Sphaerodes* isolates (Tables 3-9, and 3-10).

Another major difference with the *M. zambiae* isolate from Texas used in this study concerns the increased growth and sporulation efficiency after the addition of a filtrate from a liquid culture of the suspected host. Jordan and Barnett (1978) found that their isolate of *M. zambiae* responded positively to a mycelial extract from *F. roseum* and *Verticillium albo-atrum*. They therefore concluded that all nutrients required for growth and sporulation were contained within the living host and not from metabolites or excretions from a liquid culture.

There are also discrepancies among different studies involving required growth factors. Much of Hawker's (1938, 1939, 1950) work with fungal nutrition has found that vitamins or vitamin-like substances are often needed for optimum growth and sporulation of certain fungi. She determined that *M. destruens* (syn. *Sordaria destruens*) needed the combination of both thiamin and biotin. In addition, another vitamin-like growth factor termed mycotrophein was found to be required for *Gonatobotrys simplex* (Whaley and Barnett 1963). Jordan and Barnett (1978) determined that their isolates of *M. zambiae* also

Table 3-11. Results of the factorial study showing least square means and pairwise comparison probability values for perithecial production among pyrenomyces^a and *F. oxysporum formae speciales*^b.

	Perithecial Mean	FOB-TX Pr > [T]	FOCA-FL Pr > [T]	FOL-FP Pr > [T]	FON-LE Pr > [T]
FOB-FL	41.5	0.0001	0.0004	0.7676*	0.0001
FOB-FP	53.1	0.9427*	0.4890*	0.0001	0.0001
FOB-LE	42.9	0.0002	0.0022	0.4267*	0.0001
FOB-TX	52.9	-	0.5352*	0.0001	0.0001
FOCA-FL	51.2	0.5352*	-	0.0001	0.0001
FOCA-FP	39.0	0.0001	0.0001	0.5111*	0.0001
FOCA-LE	64.3	0.0001	0.0001	0.0001	0.2430*
FOCA-TX	52.3	0.8311*	0.6841*	0.0001	0.0001
FOL-FL	53.1	0.9252*	0.4753*	0.0001	0.0001
FOL-FP	40.8	0.0001	0.0001	-	0.0001
FOL-LE	67.1	0.0001	0.0001	0.0001	0.8757*
FOL-TX	46.3	0.0147*	0.0683*	0.0417*	0.0001
FON-FL	55.2	0.3855*	0.1370*	0.0001	0.0001
FON-FP	49.3	0.1890*	0.4876*	0.0016	0.0001
FON-LE	67.5	0.0001	0.0001	0.0001	-
FON-TX	29.4	0.0001	0.0001	0.0001	0.0001

^aFL, FP, LE = *Sphaerodes retispora* var. *retispora*; TX = *Melanospora zamiae*

^bFOB = *F. oxysporum* f. sp. *betae*; FOCA = *F. oxysporum* f. sp. *canariensis*; FOL = *F. oxysporum* f. sp. *lycopersici*; FON = *F. oxysporum* f. sp. *niveum*.

* = not significant after use of Bonferroni correction (P = 0.0031)

needed biotin and thiamin, in addition to supplemental zinc, but were capable of synthesizing their own mycotrophen. They developed a medium specific for *M. zamiae* that was glucose-based and supplemented with biotin and thiamin. It proved to be excellent for inducing maximum growth and sporulation. The current study additionally evaluated this same medium and referred to it as J&B (Table 3-1); however, it was still very poor in production of perithecia for all isolates. It was also among the worst in producing vigorous linear growth, unless supplemented with the fusarial filtrate. Conversely, Hanlin et al. (1993) and Goh and Hanlin (1994) obtained adequate results without the addition of these growth factors. Vakili (1994) found that most of his isolates of *M. damnosa* could germinate without added vitamins, but single ascospores cultures did not produce the phialidic, anamorphic state, *Gonatobotrys simplex*. However, mycelium from compatible crosses of *M. damnosa* did produce both microconidiophores and perithecia. He concluded that the absence of the phialids in single-spored cultures and their presence in crosses showed that they were spermatial in function and related to sexual reproduction. The isolates used in this study still were capable of germinating and growing without the extra nutrients although very poorly. They also formed both an anamorphic stage and perithecia from single ascospores, proving that they were homothallic.

Most physiological studies have found that nearly all fungi can utilize simple sugars such as glucose or fructose equally as well as carbon sources (Cochrane 1958, Hawker, 1950, Griffin, 1981). These simple, monosaccharides appear to be more suitable for many fungi than the more complex polysaccharides. In some instances the more complex sugars seemed less able to support hyphal growth but may be more conducive to reproduction

(Hawker 1950). Galactose is another carbon source that is apparently difficult for a number of fungi to utilize (Cochrane 1958, Hawker 1950, and Griffin 1981). Hawker (1950) determined that *M. destruens* and several species of *Chaetomium* were unable to grow vigorously with a galactose-based medium. However, the combination of good and bad carbon sources had an overall beneficial effect. For example, the addition of galactose to a glucose medium had no detrimental effect upon these fungi (Hawker, 1950). Perhaps a similar phenomenon explains the effectiveness of PDA as a growth and perithecial promoter for this group of pyrenomycetes. The combination of dextrose and starch would not be as inhibitive as starch alone, and the beneficial effect of dextrose may override that of the more complex sugar.

Several other previously published observations coincide well with the results from this study. The best media overall for the current group of pyrenomycetes tended to contain glucose or dextrose (PDA and MA), whereas the poorest contained more complex carbohydrates such as cellulose or starch (cornmeal, starch, and Emerson). Griffin (1981), points out that those fungi capable of utilizing complex carbohydrates such as starch or cellulose were also able to synthesize exocellular, digestive or hydrolytic enzymes that more readily broke the sugars down into glucose or maltose. *Melanospora* and the closely related *Sphaerodes* and *Persiciospora* are suspected of being fusion, biotrophic parasites. Organisms with this type of life strategy have additionally been determined to obtain nutrition directly through contact with their hosts without producing any cellulitic enzymes (Jeffries and Young 1994). *Sordaria* and *Chaetomium*, which were found to be unable to use galactose, (Hawker 1950) are both closely related to *Melanospora* and *Sphaerodes*. The current study concluded that Komada's agar would not allow growth of

any kind from these latter two genera. Komada's medium (Komada 1975) employs galactose as the sole source of carbon (Table 3-1), and perhaps it is partially this ingredient and not exclusively the antimicrobial supplements that are responsible for its continued, successful use as a *F. oxysporum* selective medium.

Anamorphic fungi like *Gonatobotrys*, *Gonatobotryum*, and *Phialophora* have often been linked with teleomorphic pyrenomycetes such as *Melanospora*. *Melanospora* and related genera such as *Sphaerodes* and *Persiciospora* have also been found to be ecologically associated with other fungi. Because of these close associations, many have also been presumed to be contact parasites based upon the formation of hook cells or contact branches on their hyphae. These types of fungi are also recognized to be slow-growing or incapable of growing in axenic culture. They have also been demonstrated to grow or sporulate better in mixed cultures with other organisms. Several other examples have shown enhancement of these characteristics if extracts of suspected hosts are added to media (Whaley and Barnett 1963). The current study discovered a very similar process occurring with this group of pyrenomycetes and a filtrate produced by *F. oxysporum*. The discovery of vitamin-like growth factors that improve growth and reproduction are additionally assumed to be obtained from host fungi in nature or in synthetic media insufficient for growth without added nutrients. This type of behavior has often been associated with fusion biotrophs like *Melanospora*, but was completely unknown for allied groups like *Sphaerodes*.

Although these nutritional relationships have been reported in the literature, particularly with respect to *Melanospora*, they are very few in number. No other study has attempted as extensively to address the aspect of enhancement of growth and

reproductive characteristics utilizing suspected hosts or host products. These observations have been documented from several fungal complexes, but it is still not known how common these types of interactions are outside the lab. Perhaps this type of nutritional dependency between some fungi may explain why some can be easily cultured, while others appear to be obligate.

This study has attempted to add new information that addressed *Melanospora* and other related genera specifically with *F. oxysporum*. These particular organisms and their interactions have been chosen for a number of reasons. First, these fungal complexes have been consistently found occurring together in several geographically different locations (Chapter 2). Secondly, because of the worldwide importance of *Fusarium oxysporum* and the diseases it causes, it is desirable to find novel strategies for disease management. Therefore the primary idea behind this chapter was to investigate these naturally-occurring relationships and learn how to efficiently mass produce the pyrenomycetes in the attempt to identify potential candidates for use as biological control agents for Fusarium wilt of watermelon. Chapter 4 will focus on the establishment of host ranges and the evaluation and measurement of damage to *F. oxysporum* at the bench level.

CHAPTER 4
PARASITISM AND MEASUREMENT OF DAMAGE TO *FUSARIUM OXYSPORUM*
BY SPECIES OF *MELANOSPORA*, *SPHAERODES*, AND *PERSICIOSPORA*

Introduction

The concept of parasitism can be defined or subdivided into a number of categories. Parasitism usually refers to a relationship between two organisms where one benefits at the expense of the other. However, it is not always correlated with damage or deleterious effects upon one member. Parasitic relationships can also include mutualism, in which both organisms receive equal benefit, or commensalism, where one organism benefits from the relationship, leaving the other unaffected.

Melanospora, *Sphaerodes*, and *Persiciospora* have all been found in conjunction with other fungi or from soil in field isolations (Cannon and Hawksworth 1982). All have additionally been suspected of being parasitic based upon these intimate, natural associations. Of these three genera, only *M. zamiae* has been formally investigated as a mycoparasite. Due to the formation of hook or contact cells and the ability to sporulate on living hyphal mats of *Fusarium roseum*, *Tritirachium* sp., and *Verticillium albo-atrum*, *M. zamiae* was concluded as being parasitic (Jordan and Barnett 1978). A collection of 11 other fungi, including *F. oxysporum* f. sp. *lycopersici*, were also concluded to be parasitic hosts for *M. zamiae* based solely on the hook-shaped hyphal branches formed in culture with this group of fungi. No other methods of evaluation were attempted for this second group of fungi.

It has been fully established that all three genera being used in this study were capable of more efficient growth and sporulation in dual culture with *F. oxysporum* or with some of its metabolic products in the form of a liquid culture filtrate (Chapter 3) however, this was not clear proof of parasitism. Therefore this study was initiated to thoroughly establish in a more quantitative manner if *M. zamiae* is a true mycoparasite. *Sphaerodes retispora* var. *retispora* had previously only been isolated from soil or from several different polypores (Cannon and Hawksworth 1982). *Persiciospora moreauli* has been additionally isolated from soil, and in association with several *formae speciales* of *F. oxysporum* (Cannon and Hawksworth 1982, Horie et al. 1986, Krug, 1988). However, neither of the latter two pyrenomycetes have ever been tested as parasites, thus they have been included along with *M. zamiae* in these evaluations. In addition, no other study has ever attempted to show pathogenicity or damage caused by mycoparasites on their hosts at the bench level.

The ultimate goal to this project was to find an alternative management option for Fusarium wilt of watermelon. This chapter was another step in the process of selecting potential candidates for use as biological control agents. Through the use of the pyrenomycete isolates found to be naturally occurring in the field with *F. oxysporum*, this chapter has attempted to demonstrate parasitism at the cultural level and to measure the amount of damage that these isolates cause to *F. oxysporum*. In addition to the establishment of parasitism and pathogenicity, another purpose of this chapter was to define a host range for these fungi among a collection of *F. oxysporum formae speciales*, based upon the ability to sporulate in dual culture.

Materials and Methods

Fusarium oxysporum Host Range Determination

A collection of *F. oxysporum* isolates were utilized to determine host range for the *Melanospora* and *Sphaerodes* strains. This collection consisted of both pathogenic isolates from various hosts, and non-pathogenic strains from a watermelon wilt-suppressive soil (Table 4-1). For this portion of the study, only isolates TX, FL, FP, and LE were included. Evaluations were made by monitoring perithecial production as described in Chapter III. Briefly, this entailed pairing eight mm agar plugs from actively growing cultures of *Fusarium* with cultures of *Melanospora* and *Sphaerodes* utilizing six replications per treatment. After two weeks' incubation in the dark at 25°C, four plugs were removed from each replication from the 12, 3, 6, and 9 o'clock positions at a distance of approximately two and one half cm from paired isolates. Perithecia were then enumerated from each plug with the aid of a dissecting microscope.

Two separate analyses were performed for the pathogenic and non-pathogenic isolates. Both data sets were sorted by *Fusarium* host, and analyzed by the general linear model procedure. Tests were repeated once, and means were separated by Duncan's multiple range test. The set consisting of the non-pathogenic isolates also included one pathogenic isolate for comparison.

Establishment of Parasitism

Four separate methods were tested in order to provide proof of parasitism on *F. oxysporum*. They included slide cultures, an assay using Komada's medium (KA)

Table 4-1. *Fusarium oxysporum* isolates collected from Florida and used to establish host range among isolates of *Melanospora* and *Sphaerodes*.

Isolate	Isolation Host	Plant Pathogen
1480	eggplant	Yes
1481	eggplant	Yes
1443	tomato	Yes
1453	pepper	Yes
1742	tobacco	Yes
1778	daylily	Yes
1779	pepper	Yes
2095	fern	Yes
CL58	tomato	Yes
MN21	tomato	Yes
MN27	tomato	Yes
GD8	tomato	Yes
NFL1	tomato	Yes
2099	watermelon	Yes
FO96-5	watermelon	Yes
96-2	watermelon	No
96-10	watermelon	No
3I96-1	watermelon	No
10III96-1	watermelon	No
96-6	watermelon	No
96-7	watermelon	No
96-5	watermelon	No
96-14	watermelon	No
96-17	watermelon	No
3III96-1	watermelon	No

Komada (1975) and PDA, an assay using *Fusarium* hyphal mats, and an assay using watermelon seeds previously coated with both watermelon wilt pathogen and suspected mycoparasite.

These studies consisted of *Sphaerodes* isolates FL, FP, and LE, *Persiciospora* isolate GI, and *Melanospora* isolate TX. The host being evaluated was restricted to the *Fusarium* wilt pathogen of watermelon (FON). All experiments were repeated at least once.

Slide cultures consisted of small (one mm²) agar pieces taken from actively growing cultures of both FON and suspected mycoparasites. They were mounted in water on sterile slides one half inch apart, and incubated in sterile, glass humidity chambers at 25° C. They were observed daily under a microscope for ascospore germination and formation of the hook and contact cells attaching to hyphae of *Fusarium*, as described in the literature (Jordan and Barnett 1978, Whaley and Barnett 1963).

Watermelon seeds (cultivar Charleston Gray), previously coated with a suspension of mycoparasite spores and FON were allowed to air dry in petri plates at room temperature. After 17 months, seeds were placed on moist paper towels in humidity chambers and incubated at 25° to observe growth of fungi and to additionally establish viability and survival over time of the mycoparasites.

For the hyphal mat assay, cultures of FON were grown in still liquid culture for 10-14 days. After this time, hyphal mats were removed and washed thoroughly. The mats were then placed on moist paper towels in humidity chambers and inoculated with agar plugs from the different suspected parasites. These plates were incubated at 25° C and observed periodically for growth and perithecial formation of parasites.

Lastly, a novel assay was developed that included the use of KA, one of the standard, universally-employed *F. oxysporum*-selective media, and PDA. Previous work had clearly demonstrated that *Melanospora*, *Sphaerodes*, and *Persiciospora* were all completely inhibited on this medium, whereas PDA was one of the better media for growth and sporulation (Chapter 3, Harveson and Kimbrough 1998). A sterile 40 mm diameter beaker was placed in the center of a petri plate and molten PDA was poured around the outside. After the agar solidified, the beaker was removed, leaving a 40 mm diameter hole in the middle ringed by PDA. The hole was gently filled with KA and allowed to harden. Agar plugs of FON and all suspected parasites were paired on the center portion of plates containing the KA and incubated at 25° C (Fig. 4-1).

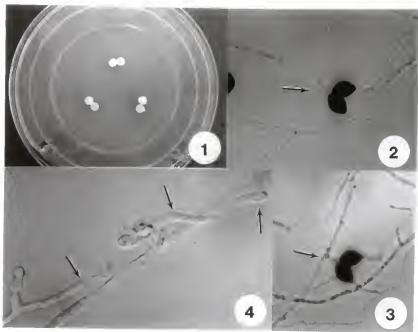
Measurement of Deleterious Effects of Parasites on *F. oxysporum*

Three separate techniques were developed to not only provide evidence of deleterious effects on FON by parasites at the cultural level, but more importantly, to measure the damage inflicted by *Melanospora*, *Sphaerodes*, or *Persiciospora*. The measurement of damage in vitro to *Fusarium* was accomplished with several categorical assays, including hyphal weight reduction, growth inhibition, and reduction of aerial hyphae.

The hyphal weight reduction tests were performed in large petri plates (100 x 150 mm) using liquid culture (PD broth). A standard volume of 15 ml was put in each plate along with eight mm agar plugs of various *Fusarium* and parasite treatments. The isolates utilized for this study consisted of the four primary *formae speciales* evaluated in Chapter

Figures 4-1-4-4. Assays to determine parasitism of *Fusarium oxysporum*

- 4-1. Assay plates showing both parasite and *Fusarium* co-inoculated in center portion of plates containing the Komada medium. Outside ring contains PDA (x 0.6)
- 4-2. Germinating ascospores showing attachment of parasite germ tubes on *Fusarium* hyphae (x 500)
- 4-3. Germinating ascospores showing attachment of parasite germ tubes on *Fusarium* hyphae (x 500)
- 4-4. Hyphal strands of parasite attaching to *Fusarium* hyphae and forming contact cells (arrows) (x 1100)



3, isolates FL, FP, LE, and TX and the corresponding pyrenomycete found in association with each *forma specialis*, FOCA, FOL, FON, and FOB, respectively. Each *Fusarium forma specialis* was grown by itself, which served as the control, and with each of the four suspected parasites in all possible combinations. Each treatment consisted of five replications. After five days' incubation at 25° resulting hyphal mats were filtered through cheesecloth, squeezed free of liquid and weighed. Dry weights were additionally obtained after air-drying hyphae for several days in petri plates. Data from hyphal weights were analyzed by the general linear model procedure and means were separated by Duncan's multiple range test.

Reduction of aerial hyphae was evaluated on *F. oxysporum* f. sp. *niveum* only. The various parasites were paired six cm apart on PDA and MA plates utilizing six replications per assay. These plates were compared to FON growing singly on the same media. Linear growth of FON for all medium treatments was measured daily for 12 days. Collected data were analyzed as a repeated measures experiment and additionally subjected to linear regression using the simple regression model: $y = a(\text{trt}) + b(\text{obs})$, where the y equaled growth response and a and b were parameters to be estimated.

The growth inhibition assay was evaluated in a similar manner to that described above for aerial hyphal reduction. This assay consisted of the same isolates, media, and replications, only the mycoparasite was first put on plates to grow singly for three days. After this time, FON was placed at the edge of the parasite colony. Linear growth of FON was then measured as before on a daily basis. Data were similarly analyzed as a repeated measures experiment before being subjected to linear regression. This assay employed the quadratic model: $y = a(\text{trt}) + b(\text{obs}) + c(\text{obs}^2)$, where y = growth response

and a, b, and c were parameters to be estimated. Both of these two assays were repeated once and results were separated by the least square means procedure. Pairwise probability values obtained were additionally adjusted with the Bonferroni correction.

Results

The results of the host range determinations are presented in Tables 4-2 and 4-3. Table 4-2 shows the production of perithecia by *Melanospora* and *Sphaerodes* in conjunction with the pathogenic isolates of *F. oxysporum*. There was no one parasite isolate that statistically predominated over the others with respect to reproductive ability. Numerous differences were observed between and among isolates with all four parasites being represented at least once as being the best to sporulate on a fusarial isolate. Overall, isolates LE and FL tended to produce higher numbers of perithecia than did FP or TX. The data in Table 4-3 illustrate a similar story regarding differences among and between parasite isolates and their abilities to efficiently produce perithecia on the non-pathogenic isolates. However, large differences were observed in total perithecial production between pathogenic and non-pathogenic isolates. There were no *Fusarium* isolates among those tested that did not support at least some sporulation from the parasites. With the exception of 96-14, sporulation for all parasites was much poorer on the non-pathogenic isolates. This can be easily visualized by observing the one pathogenic isolate analyzed with the rest in Table 4-3, and by also comparing overall values between Tables 4-2 and 4-3. The recognition that isolate 96-14 was capable of supporting good perithecial production was an important observation and will be implemented and discussed further in Chapter 5.

Table 4-2. Results of host range study for pathogenic isolates of *F. oxysporum* using isolates of *Melanospora* and *Sphaerodes*

<i>Fusarium</i> isolate	Parasite Isolate ^a			
	FL ^{b,c}	FP	LE	TX
1443	60.1b	59.7b	72.6a	43.2c
1453	70.2a	33.7c	46.9b	0.0d
1480	49.1c	34.4d	72.2b	82.3a
1481	43.2bc	36.6c	51.9b	68.3a
1742	59.9a	53.6a	60.2a	54.4a
1778	64.0a	51.6a	56.1a	51.7a
1779	63.1a	67.0a	66.3a	41.2b
2095	33.5a	32.3a	40.2a	4.3b
2099	49.1c	59.7bc	77.9a	70.1ab
CL58	57.3b	41.1c	75.2a	78.1a
GD8	65.0a	58.7a	63.0a	37.9b
MN21	83.0a	66.5b	82.1a	52.8b
MN27	78.9a	66.1a	79.1a	43.1b
NFL1	70.1a	63.1a	60.9a	34.8b

^aFL, FP, and LE = *Sphaerodes retispora* var. *retispora*; TX = *Melanospora zamiae*

^baverage number of perithecia produced by parasite isolates

^cMeans followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.01)

Table 4-3. Results of the host range study for non-pathogenic isolates of *F. oxysporum* using isolates of *Melanospora* and *Sphaerodes*.

<i>Fusarium</i> Isolate	<i>Parasite Isolate</i> ^a			
	FL	FP	LE	TX
10III96	4.0b ^{b,c}	33.7a	29.6a	15.0b
3I96-1	26.6a	34.9a	3.0b	8.0b
3III96-1	26.2a	34.5a	25.9a	35.5a
96-10	22.0b	24.5b	47.6a	2.0c
96-14	72.5a	79.1a	88.6a	87.5a
96-17	26.9a	34.6a	36.1a	12.6b
96-2	23.7a	25.5a	15.4a	19.3a
96-5	24.9a	21.1a	18.1a	16.5a
96-6	18.7ab	5.0b	26.5a	7.0b
96-7	9.7b	29.9a	32.1a	24.2a
FO95-5*	76.0b	64.7b	96.5a	44.2c

^aFL, FP, and LE = *Sphaerodes retispora* var. *retispora*; TX = *Melanospora zamiae*

^baverage number of perithecia produced by parasite isolates

*= pathogenic isolate of *F. oxysporum* used for comparison

^cMeans followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.01)

The slide cultures for all isolates produced similar results to those in the literature describing mycoparasitism by *M. zamiae*. The isolates of *Persiciospora* and *Sphaerodes* reacted in an identical manner. All three genera germinated and formed contact cells or hooks on their hyphal strands that appeared to make contact with mycelial branches of the *Fusarium* isolates. The ability to form these specialized branches was apparently not host specific for each parasite and its corresponding field host. Each *forma specialis* appeared to be equally receptive to any of the parasites that were evaluated. These phenomena of germination and attachment of ascospores and hyphae are demonstrated in Figures 4-2, 4-3 and 4-4.

The watermelon seeds coated with both organisms simultaneously produced the same results. After several weeks, perithecia were observed on the outer edges of the paper towels in the humidity chambers, but no fusarial growth could be visualized. Perithecia were removed from the periphery of the chambers and plated on media. Resulting growth of both organisms indicated that the parasites were colonizing FON as it grew saprophytically on the paper towel substrate. The hyphal mat assay produced the same results. The isolates of *Sphaerodes* and *Melanospora* sporulated profusely on the FON mats only, but not on the paper towels in the humidity chambers. The controls for both assays consisted of parasite isolates placed singly on paper towels in chambers, and did not result in perithecial formation. Therefore, it was concluded that the *Sphaerodes* and *Melanospora* isolates were growing on and parasitizing FON due to the lack of any additional nutritional source in the humidity chambers.

The assay consisting of KA and PDA provided the final and perhaps most convincing evidence for the parasitic abilities of these fungi with *F. oxysporum*. All three

genera were evaluated against FON for this test. Representatives of each (LE-*Sphaerodes*, TX-*Melanospora*, and GI-*Persiciospora*) are illustrated in Figures 4-5-4-7. In all cases, scattered perithecia were formed after several weeks on the central portion of the plates containing the KA however there were differences in the ability to sporulate with *Sphaerodes* forming the most perithecia followed by *Melanospora* and *Persiciospora*. The outer ring of PDA in all examples consisted of a layer of perithecia greater than that on the KA (Fig. 4-8). Komada agar was previously shown to inhibit growth and sporulation for these isolates. However, perithecia formed on this normally restrictive medium in conjunction with FON, and reproduction also was observed at points removed from the initial inoculation site on the portion containing PDA. Therefore, it was concluded that the most logical explanation for these events was the occurrence of parasitism of *Fusarium* by the isolates of *Sphaerodes*, *Melanospora*, and *Persiciospora* as it grew outward from the center.

The attempt to provide evidence for damage to *F. oxysporum* in culture was first done by measuring the total hyphal weights of the various *formae speciales* alone and in combination with the different parasites. Table 4-4 demonstrates the reductive effect on *Fusarium*, but it includes only the four *formae speciales* and the corresponding parasites originally found with them from the field. Although these evaluations were done with all parasites and all *formae speciales* in all possible combinations, there were not many differences between the treatments containing both fungi. However, the important point to consider is that both fungi together produced hyphal weights that were significantly

Figures 4-5-4-10. Results of *Fusarium* parasitism and pathogenicity assays.

- 4-5. *Sphaerodes retispora* var. *retispora* (LE) parasitizing FON. Note heavier concentration of perithecia in outer ring of PDA. (x 0.67)
- 4-6. *Melanospora zamiae* (TX) parasitizing FON. Note heavier concentration of perithecia in outer ring of PDA, and the fewer number of perithecia overall compared to that of LE. (x 0.67)
- 4-7. *Persiciospora moreau* (GI) parasitizing FON. Note the fewer number of perithecia overall compared to both LE and TX. (x 0.68)
- 4-8. Close-up view of parasitism of FON. Shows scattered perithecia in center portion of plate with heavier concentration on outside ring of PDA. Arrow depicts the line separating the two different media. (x 0.58)
- 4-9. Plate showing the reduction of *Fusarium* aerial hyphae. Single arrow depicts the final advance of FON before being reduced. Double arrows shows the extent of FON growth when picture was taken (x 0.83)
- 4-10. Plate showing the inhibition of *Fusarium* hyphae. Note the clear line of inhibition where the parasite stops FON growth (x 1.0)

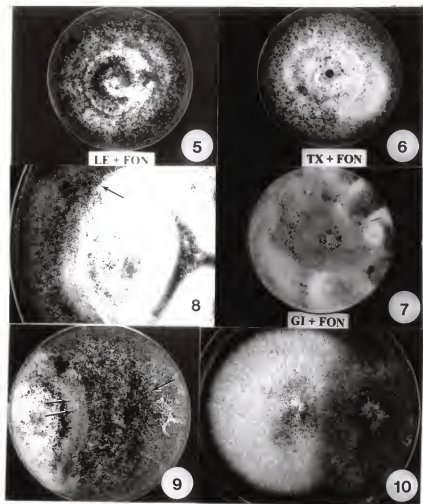


Table 4-4. Reduction of total hyphal weights of *F. oxysporum formae speciales* by each parasite originally found with them.

Treatment ^a	Wet weight (g)	Dry weight (g)
FOCA	0.83 a ^b	0.145 a ^b
FOB	0.75 b	0.135 b
FON	0.73 bc	0.13 bc
FOL	0.69 c	0.12 c
FOCA + FL	0.42 d	0.075 d
FON + LE	0.33 ef	0.057 ef
FOB + TX	0.32 ef	0.055 ef
FOL + FP	0.31 ef	0.055 ef

^aFOCA = *F. oxysporum* f. sp. *canariensis*; FOB = *F. oxysporum* f. sp. *betae*; FON = *F. oxysporum* f. sp. *niveum*; FOL = *F. oxysporum* f. sp. *lycopersici*

FL, FP, and LE = *Sphaerodes retispora* var. *retispora*; TX = *Melanospora zamiae*

^bMeans followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.01$).

lower than any of the *formae speciales* alone. This demonstrates that these four parasites were pathogenic and caused measurable damage to the particular *forma specialis* with which they were isolated. Each also induced highly significant weight reductions to the other *formae speciales* as well (data not shown), but very few differences were observed between these different parasite treatments.

The hyphal reduction test did not initially look very promising. Until about the sixth day of taking measurements, the fusarial growth on all parasite treatments was equal to that of the FON control. However, after this time, the hyphal growth of FON began to recede and appeared to melt away. This occurred on the aerial growth only, one could still see the mycelium on the agar surface. As the hyphae of *Fusarium* receded, the parasite growth advanced in the same direction and perithecia were readily formed in the process (Fig. 4-9). Table 4-5 exhibits the average growth of FON after 12 days for each treatment. Isolate LE caused the most reduction to FON, but was no different statistically than isolates FL or FP. Because these three treatments were statistically different than that of FON alone, it was concluded that they significantly reduced the amount of aerial mycelium compared to the control. This was interpreted to mean that they were pathogenic to FON. The remaining two isolates, GI and TX, were not different statistically than FON. Therefore, they were not causing distinguishable damage to FON by the reducing aerial hyphae.

The knowledge gained from these prior observations led to the development of another, slightly different assay. The growth reduction test entailed growing the parasites on media for three days prior to the addition of FON. The final results were similar to

Table 4-5. Reduction of aerial hyphae of *F. oxysporum* f. sp. *niveum* by *Persiciopsora*, *Melanospora*, and *Sphaerodes*.

Treatment ^a	LS Mean (mm)	FON	FL	FP	GI	LE	TX
		Pr > [I]	Pr > [I]	Pr > [I]	Pr > [I]	Pr > [I]	Pr > [I]
FON	47.5	-	0.0001	0.0001	0.2868*	0.0001	0.2868*
FL	41.6	0.0001	-	0.7147*	0.0001	0.0063*	0.0001
FP	42.0	0.0001	0.7147*	-	0.0001	0.0025*	0.0001
GI	48.6	0.2868*	0.0001	0.0001	-	0.0001	0.0342*
LE	38.9	0.0001	0.0063*	0.0025*	0.0001	-	0.0001
TX	46.6	0.2868*	0.0001	0.0001	0.0342*	0.0001	-

^aFON = *F. oxysporum* f. sp. *niveum*; FL, FP, and LE = *Sphaerodes retispora* var. *retispora*; GI = *Persiciopsora moreau*; TX = *Melanospora zamiae*

* = not significant after use of the Bonferroni correction ($P = 0.0016$)

Table 4-6. Growth inhibition of *F. oxysporum* f. sp. *niveum* by isolates of *Persiciospora*, *Melanospora*, and *Sphaerodes*.

Treatment ^a	LS Mean (mm)	FON	FL	FP	GI	LE	TX
		Pr > [T]	Pr > [T]	Pr > [T]	Pr > [T]	Pr > [T]	Pr > [T]
FON	50.8	-	0.0001	0.0001	0.0024*	0.0001	0.0001
FL	47.3	0.0001	-	0.0955*	0.0001	0.0031*	0.7202*
FP	46.4	0.0001	0.0955*	-	0.0001	0.1898*	0.1898*
GI	49.0	0.0024*	0.0045*	0.0001	-	0.0001	0.0014
LE	45.6	0.0001	0.0031*	0.1898*	0.0001	-	0.0091*
TX	47.1	0.0001	0.7202*	0.1898*	0.0014	0.0091*	-

^aFON = *F. oxysporum* f. sp. *niveum*; FL, FP, and LE = *Sphaerodes retispora* var. *retispora*; GI = *Persiciospora moreau*; and TX = *Melanospora zamiae*

* = not significant after use of the Bonferroni correction (P = 0.0016)

the aerial hyphal reduction test, but were more visually spectacular (Fig. 4-10). The data from this assay are presented in Table 4-6. Like the previous test, isolate LE was the most aggressive and effective in halting fusarial growth. However, it was again no different statistically from isolates FL or FP. The major difference between these two inhibition assays was observed with isolate TX. Unlike its performance in the aerial reduction TX was effective in inhibiting FON, and was statistically insignificant from the pathogenic isolates LE, FL, and FP (Table 4-6).

Discussion

Jordan and Barnett (1978) tested over 20 different fungi, and found that 11 were judged to be hosts for *M. zambiae* by their evaluation method of observing contact cell formation. This group included the tomato wilt pathogen, *F. oxysporum* f. sp. *lycopersici*, but no study has ever exclusively evaluated many different form species of *F. oxysporum*. There has also never been any type of parasitic host ranges established for *Sphaerodes*. The results obtained in this study for host range determination were based upon the ability to sporulate on the various *formae speciales*. They revealed a large number of differences among and between the parasites and their reproductive abilities. This type of information is not really very surprising, because variation in biological characteristics is a common phenomenon in mycology.

Chapter 3 found that the parasites differed significantly in the ability to sporulate on different media in the absence of *F. oxysporum*. It further demonstrated that production of perithecia was better for all parasites in the presence of *F. oxysporum*. In general, it was shown that *M. zambiae* tended to sporulate less than *Sphaerodes*, regardless

of whether *F. oxysporum* was present or not (Chapter 3). This is an interesting finding in light of the fact that the species of *Sphaerodes* studied had never been found with *F. oxysporum* (Cannon and Hawksworth 1982). However, after a larger number of *formae speciales* was tested, *M. zamiae* proved to have some clear host preferences in terms of producing perithecia. These included isolates 1480 and 1481 (Table 4-2). It additionally was among the poorest at reproducing on others like 1443, 1453, and most of the non-pathogenic isolates (Tables 4-2 and 4-3). This suggests that ability to sporulate by these groups of fungi is not simply a matter of some being able to and some not, but that other factors like the presence of an adequate host also plays a role. It also suggests that ability to sporulate may be an excellent criterion to initially judge parasitism and host-parasite relationships. The host range study has also demonstrated that no one parasite is going to be effective as a biological control agent for every *F. oxysporum*. There will be a need for testing potential biological agents in a similar manner as this in order to determine the most likely candidate.

This study has brought into question the validity or accuracy of establishing parasitism by relying strictly on the formation of the specialized contact hyphal branches of the suspected parasites. Although this is obviously an important finding, there should be other methods tried in combination to completely address this question. This chapter has combined the traditional technique with newly developed ones in the attempt to provide more conclusive evidence for parasitism among this group of pyrenomycetes on *F. oxysporum*. Taken together, these methods have strongly indicated that the fungi found occurring naturally with *F. oxysporum* in the field have the capacity to function as parasites.

Another important feature of parasitism has further been discovered. Just because an organism may sporulate well on, or be able to parasitize its host, this is not always a good indication that it will be an effective agent for biological control. All of the genera from this study were demonstrated to be parasitic on the watermelon wilt pathogen but there were different levels of damage caused by these organisms. *Persiciospora moreau* was isolated in conjunction with FON (Chapter 2), and was shown to be parasitic in all tests in which it was evaluated. However, the pathogenicity assays (reduction of aerial hyphae and growth inhibition) indicated that deleterious effects upon FON by *P. moreau* were negligible (Tables 4-5 and 4-6). This suggests that it functions as a mutualistic or commensalistic parasite, but not as a pathogen.

This chapter has been the first attempt to demonstrate damage in culture by mycoparasites on their hosts, and has shown that *F. oxysporum* is an excellent host for *Sphaerodes retispora* var *retispora*. *Sphaerodes* is actually more aggressive and harmful on *Fusarium* than the previously-studied parasite, *M. zambae*. This also represents the first definitive proof of parasitism for both *Sphaerodes* and *Persiciospora*, as well as the first report of *P. moreau* in the western hemisphere.

The next chapter will focus on the selection of one of these parasites as a candidate for biological control based upon all information gathered throughout the entire study. It will then describe the methods and techniques used for evaluating the ability to reduce disease severity on watermelon by the *Fusarium* wilt pathogen at the greenhouse level.

CHAPTER 5

ECOLOGY AND USE OF *SPHAERODES RETISPORA* VAR. *RETISPORA* FOR THE CONTROL OF FUSARIUM WILT OF WATERMELON

Introduction

The Fusarium wilt and root rot diseases induced by *formae speciales* of *Fusarium oxysporum* are among the most severe plant diseases in the world. They cause significant economic losses to a wide range of species and occur on plants belonging to all families except the Gramineae (Alabouvette et al. 1996).

These fungal diseases are some of the oldest recognized and among the most difficult to manage as well. Most control methods available today are either inefficient or difficult to apply. Chemical fungicides are ineffective because of the location of the pathogen in the plant's vascular system. Fumigation can be successful, but is erratic and often cost-prohibitive, depending upon the value of the crop. The beneficial effects it does produce are only temporary and must be reapplied each season due to the pathogen's propensity to rapidly recolonize soils (Marois and Mitchell 1981). Short term crop rotation is of little value because *F. oxysporum* can colonize non-host plants and survive as chlamydospores for long periods of time. Therefore, genetic resistance is generally considered to be the most effective strategy for managing wilt diseases. However, this can be a difficult procedure if no known resistance genes are available, or if many different pathogen genotypes are present, necessitating the need for germplasm containing multiple resistance genes.

Fusarium wilt of watermelon, caused by *F. oxysporum* f. sp. *niveum* (FON), is a major limiting production factor wherever watermelons are grown. This disease was first reported in 1894 (Smith) from Georgia and South Carolina, but few methods have been developed since that time that consistently reduce disease severity and yield losses. Biological control, in the form of suppressive soils has been recognized for over 70 years (Cook and Baker 1983), but has received a great deal of attention over the last 10-15 years. *Fusarium*-suppressive soils have been identified and intensively studied in several locations throughout the world. However, with the exception of a watermelon wilt-suppressive field in central Florida (Hopkins and Elmstrom 1987), most appear to be constitutive and are unlikely to be artificially created from a conducive soil. They all additionally appear to have different mechanisms of suppression, so no one suppressive site would be consistent with any other in terms of generating standard suppressive-soil characteristics or control recommendations. Biological control utilizing mycoparasites has also been gaining in popularity and usage, but none have proven to be successful against *F. oxysporum*.

The discovery of a group of pyrenomycetous found to be consistently occurring with several different *formae speciales* of *F. oxysporum* (Chapter 2), has generated the interest to test these organisms as potential biological agents for the Fusarium wilt disease of watermelons. Chapter 3 showed that *Melanospora*, *Sphaerodes*, and *Persiciospora* were all strongly dependent upon *Fusarium* for enhanced growth and sporulation as well as being truly mycoparasitic. It was further demonstrated in Chapter 4 that *Melanospora* and *Sphaerodes* caused measurable damage to the watermelon wilt pathogen at the cultural level. Based on the information learned throughout this entire study, isolate LE,

identified as *Sphaerodes retispora* var. *retispora*, was selected as the candidate for further studies concerning disease reduction at the greenhouse level. This isolate was chosen because of its ability to effectively sporulate in conjunction with the wilt pathogen, and consistently be among the best at reducing growth parameters of FON in the pathogenicity assays. Perhaps the most important criterion for the selection of this isolate was the fact that it was naturally found in that *Fusarium*-suppressive field in Leesburg in association with a pathogenic isolate of *F. oxysporum* f. sp. *niveum*. Several ecological aspects concerning this isolate were additionally investigated, including survival over time in soil, ability to colonize watermelon roots, and ability to reduce *Fusarium* propagules in soil.

Materials and Methods

Preparation of sodium alginate pellets

Before evaluating the efficacy of *S. retispora* for disease control, a practical delivery system needed to be developed and tested. All portions of this study utilized the encapsulation of isolate LE into sodium alginate pellets. This technique was accomplished with minor modifications of those described in previously published reports (Fravel et al. 1985, Lewis and Papavizas 1985, 1987). It included a suspension of LE ascospores and hyphal fragments mixed with an aqueous solution of ground wheat germ and sodium alginate.

Specifically, 10 g of sodium alginate were dissolved into 375 ml of water. Twenty-five g of ground wheat germ were mixed with 125 ml of water in a separate flask. Two to three week old cultures (5) of isolate LE were ground with 200 mls of water in a blender. The flasks containing the wheat germ and sodium alginate were sterilized in an

autoclave and allowed to cool to room temperature. The final aqueous solution was comminuted once more in a blender, and consisted of 300 mls sodium alginate, 100 mls wheat germ, and 100 mls isolate LE. The final solution was placed into an aspiration flask, continuously stirred on a stir plate and dripped through a Pasteur pipette into a beaker containing 0.25 M CaCl_2 . As each drop of the final solution hit the CaCl_2 solution, it was encapsulated into a spherical bead or pellet. Theoretically, the sodium in the alginate solution is displaced by the calcium in the receiving solution, and each pellet should contain equal amounts of all solution ingredients (Fravel et al. 1985). After the entire 500 mls of final solution was run through the apparatus, pellets were washed several times with water, and stored afterwards in the refrigerator in glass bottles.

Pellets were viewed under a compound microscope to observe numbers of incorporated ascospores. They were also plated onto media periodically to confirm viability of the *Sphaerodes* isolate, and presence of contaminants. This same procedure was performed for other treatments to be evaluated, including pellets containing a non-pathogenic *F. oxysporum* isolate (isolate 96-14), isolate 96-14 combined with isolate LE, and pellets alone with no microorganisms in the solution.

Delivery system methods

Four separate methods for evaluation of disease protection were attempted. The first consisted of mixing pellets with commercial potting soil at a rate of five g pellets to one liter of potting soil (w:v). This mixture was placed into Speedling[™] trays and watermelon seeds (cv. Charleston Gray) were planted into the cells. Plants were grown for four-six weeks, roots were removed, washed free of soil material, and dipped into a microspore suspension (1×10^6 conidia/ml) of FON, isolate CSI-24, and transplanted into

potting mix in pots using three plants per replication with six replications per treatment. All subsequent tests included this isolate as the pathogen. This first evaluation consisted of only four treatments, two with isolate LE pellets and two without. The pathogen was inoculated on one treatment with pellets and one without. After eight weeks' incubation in the greenhouse, entire plants (all roots and foliage) were removed, washed, dried in paper bags, and weighed. Later evaluations consisted of six treatments, including healthy control, pathogen control, pelleted isolate LE, pelleted isolate LE and 96-14, pelleted isolate 96-14 alone, and pellets alone. All treatments except for the healthy control were root-inoculated with the pathogen after four-six weeks, and evaluated as described above after an additional eight weeks incubation.

The remaining three methods all utilized soils infested with the pathogen at approximately 250 propagules per g of soil. Soils used for all experiments were collected from non-cultivated land, and were not sterilized. Six week old plants grown in ordinary potting soil in speedling trays were washed free of organic material and root-dipped into suspensions of each of the six treatments described above. They were then transplanted into ten cm clay pots infested with FON. Degree of infestation was determined by dilution platings. After six to eight weeks, dead plants were recorded and plants were removed, dried, and weighed.

Two additional methods were attempted that utilized the alginate pellets. One of these consisted of mixing pellets in potting soil and planting seeds in Speedling[™] trays. After four to six weeks, they were removed, washed, and transplanted into infested field soil in the clay pots. The other consisted of mixing pellets into infested field soil in a ratio of five g of pellets per replication and watering daily for a duration of one week. Each pot

contained approximately 300 g of soil. Six week old seedlings were then transplanted into the pots. Both these last two methods were treated in the same manner as described before by taking mortality counts and dry weights after six to eight weeks.

Since the efficacy of the *Fusarium* wilt-suppressive field in Leesburg, FL had been extensively documented and studied, (Hopkins and Elmstrom 1987, Larkin et al. 1993, 1996) it was also desired to compare the different treatments of *Sphaerodes* to both conducive and suppressive soils from this field. The idea was to test whether isolate LE would give any additional benefit above that provided by the suppressive soil alone, or if it would contribute to reducing the harmful effects of the conducive soils. Soils from both suppressive and conducive plots in this field were collected, and mixed with sodium alginate pellets at 5 g per replication and planted with watermelon seedlings like that previously described. These evaluations consisted of eight treatments, suppressive and conducive soils alone, and each with pellets containing isolate LE, isolate LE plus the non-pathogenic 96-14 isolate, and the 96-14 isolate alone. All tests evaluating these four methods were repeated at least once

Ecological studies

Finally, since virtually nothing was known about the ecology of *Sphaerodes* or its role as a soil inhabitant, some experiments were undertaken to determine how it reacted in response to the watermelon plants and the pathogenic isolate of *Fusarium*. Watermelon seedlings were root-dipped with a suspension of isolate LE, or LE in combination with non-pathogenic isolate 96-14, and transplanted. Seeds were also planted into soil mix containing the pelletized isolate LE and allowed to incubate for several months. After

several months, plants were removed, washed, and plated on various media to try to determine if *Sphaerodes* had been able to colonize watermelon roots.

To determine soil survivability, unsterilized field soils were inoculated with pellets containing isolate LE, and one half were planted with seeds, leaving the other half without plants. These pots were maintained in a walk-in growth chamber for a period of nine months (June-February) to mimic the normal time period between watermelon plantings in central Florida. Soil dilutions from these pots were performed and plated on media to simply establish viability of isolate LE, both in the presence and absence of plants. Because of the faster growth of various soil saprophytes, additional measures were required to assist the identification of *Sphaerodes* (Malloch 1970). This included suspending soil samples in 50% ETOH and stirring for 5 minutes. One ml was pipetted into plates before the addition of molten media. After solidification, plates were incubated at 40° C for 24 hours before being transferred to an 18° incubator. This procedure eliminated many imperfect fungi and bacteria.

Lastly, clay pots were filled with soil infested with approximately 250 propagules of *F. oxysporum* per gram, and mixed with both pelletized isolate LE and suspensions the fungus consisting of both ascospores and hyphal fragments. They were compared to soils containing only the wilt pathogen. These evaluations were done both with and without the presence of watermelon plants as before. After two to three months, dilution platings were done on Komada's medium (Komada 1975) in order to monitor change in FON propagule levels. The purpose of this experiment was to determine if the *Sphaerodes* isolate was capable of directly attacking or reducing fusarial chlamydospores in soil.

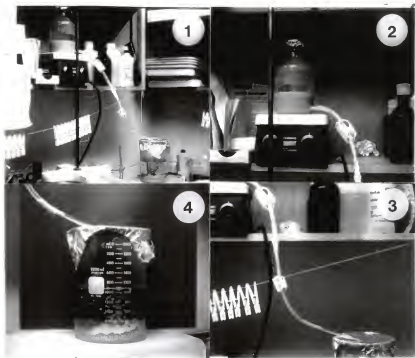
Results

The sodium alginate pellets proved to be an adequate system for incorporating the isolate of *Sphaerodes*, and delivering it to its desired destination. Figures 5-1-5-4 show the apparatus that was constructed for producing the alginate pellets. Periodic plating of the pellets on media resulted in growth whenever it was attempted. Refrigeration of the pellets retained viability of the isolate for at least two months. They were never tested after this period, and new batches of pellets were routinely produced on this approximate time scale. Pellets observed under the microscope contained 10-15 ascospores per field of view using the 10x power objective, and was consistent with each batch as long as the cultures used were consistently the same age.

The potential for disease reduction was investigated first. After plants were exposed to the *Sphaerodes* isolate for six weeks, they were root dipped in a FON spore suspension and transplanted. None of the plants died from this experiment, but there were significant differences in their total dry weights (Table 5-1). The first two tests evaluated isolate LE alone, but was not included in the last experiment because it caused no apparent pathogenicity to the plants. These four treatments just before harvesting are illustrated in Figure 5-5. Statistically, plants treated with *Sphaerodes* were no different in size than the healthy controls. (Table 5-1). Therefore, this treatment was included in test 3 to determine if the pellets alone had any effect on disease reduction. It was also desired to preliminarily test the addition of a non-pathogenic isolate of *F. oxysporum*, alone and with isolate LE because they would be included in later delivery method evaluations.

Figures 5-1-5-4. The apparatus used for the encapsulation of isolate LE in sodium alginate pellets (x 0.25).

- 5-1. The entire system with all components in place as pellets are being produced.
- 5-2. The aspiration flask containing the final solution being continuously stirred on a stir plate.
- 5-3. Final solution flowing down tube from flask to beaker containing CaCl_2 receiving solution
- 5-4. Final solution dripping into receiving solution of CaCl_2 . Pellets are formed as sodium in final solution is displaced by calcium in receiving solution.



Figures 5-5-5-6. Effects of isolate LE on disease reduction of FON on watermelon.

- 5-5. Plants exposed to isolate LE in transplant mix for six weeks and inoculated by a microspore suspension of FON. Reading from left to right: healthy control, FON-inoculated control, LE-inoculated with FON, and LE alone (x 0.125).
- 5-6. Plants exposed to isolate LE in transplant mix for six weeks and transplanted into FON-infested soils. Reading from left to right: healthy control, FON control, LE alone, non-pathogenic isolate 96-14 alone, and LE combined with 96-14. All treatments were transplanted into infested soils except healthy control (x 0.125).



Table 5-1. The effect of isolate LE on protection of watermelon plants after inoculation of treated roots with a microspore suspension of *Fusarium oxysporum* f. sp. *niveum*.

Treatment	Test 1 dry wt (g)	Test 2 dry wt (g)	Test 3 dry wt (g)
Control	4.26ab ^a	4.67a	4.05a
LE	5.60a	5.23a	-
LE+FON	3.50b	5.10a	3.65b
FON	1.12c	0.55b	0.67e
LE+96-14	-	-	1.93c
96-14	-	-	0.77de
Pellets	-	-	1.11d

^aEach value is the average of 18 plants (three plants per replication)

Means followed by the same letter and not significantly different according to Duncan's multiple range test ($P = 0.01$).

The results in Table 5-1 indicate that isolate LE was not causing any damage to the watermelon plants. In all tests, there was some level of protection being given to the plants after challenging with the pathogen, and in one of the three tests it was no different statistically from the healthy control. In the other two tests it was intermediate between the positive and negative controls. It was also noted in tests 1 and 2 that the LE treatment appeared to induce some added growth benefit, but it was not known if this was a response to the alginate or wheat germ added in the pellets or to LE itself. It was also not known whether the apparent protection from the pathogen was provided by isolate LE, or to some form of resistance due to better and more rapid growth by plants. Therefore the third test was conducted with pellets added alone, and it was established that this treatment afforded little if any protection from the pathogen (Table 5-1).

These results were encouraging, but were not very realistic in terms of disease evaluation. Thus, three different delivery methods were developed that used FON-infested soils as inoculum. These methods would more accurately reflect a field situation. The data from these test are presented in Table 5-2. Methods 2 and 3 proved to be statistically better than method 1 regarding percent mortality (data not shown), but not many differences were observed between the three in terms of plant dry weights. Visual effects of these treatments after three week's incubation are shown in Figure 5-6. However, root dipping plants with the biocontrol fungus is not a practical way of introducing a biological control agent, so it was good to observe that methods using the pellets were equally as successful. It is interesting to note here that the three new treatments (those involving pellets alone and isolate 96-14) did not result in better yields.

Table 5-2. Evaluation of delivery system methods for reduction of disease caused by *F. oxysporum* f. sp. *niveum* in infested soils.

Treatment ^a	Method 1 ^b		Method 2		Method3	
	% death	dry wt (g)	% death	dry wt (g)	%death	dry wt (g)
Control*	0.0a ^c	1.35a	0.0a	2.0a	0.04a	1.32a
FON	25.0b	0.18c	66.6c	0.40c	50.0b	0.26c
LE	0.0a	0.61b	25.0ab	1.70a	0.04a	0.86b
LE+96-14	0.0a	0.39c	25.0ab	0.84b	58.3b	0.47c
96-14	50.0d	0.19c	41.6bc	0.37c	58.3b	0.22c
Pellets alone	33.3c	0.21c	41.6bc	0.54bc	58.3b	0.28c

^aFON = *Fusarium oxysporum* f. sp. *niveum*; LE = *Sphaerodes retispora* var. *retispora*; 96-14 = non-pathogenic isolate of *F. oxysporum*

^bMethod 1 = root dip plants with treatment suspensions; Method 2 = mix pellet treatments in potting soil and expose to plants via seeding into mixture; Method 3 = mix pellet treatments into FON-infested soils

^cEach value is the average of 24 plants (two plants per replication)

Means followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.01$).

* = Only treatment that was not inoculated with FON

The pellets by themselves, again provided no added protection, nor did the two treatments including the non-pathogenic isolate 96-14.

The results from the suppressive soil evaluations are included in Table 5-3. They indicate that the addition of isolate LE to soils prior to planting resulted in mortality counts and plant dry weights that were equal to or better than the naturally suppressive soil. According to the mortality data involving the treatment with 96-14 and the suppressive soil, it looks like this isolate may be providing some protection of resistance to the plants. However, the dry weights for this treatment are very poor, and are no different than the worst treatments utilizing the conducive soils. These results suggest that the observed benefit regarding total plant weights and percentage of dead plants are being provided by the *Sphaerodes* isolate and not by the non-pathogenic isolate 96-14.

The ecological data obtained are difficult to interpret. It was clearly established that isolate LE was capable of long-term survival in soil. It was always found in dilution platings from soil after three to four weeks. This helped demonstrate that the pellets could effectively deliver spores of this fungus. It also showed that LE could remain viable after at least nine months in soils subjected to constant wetting and drying and fluctuating temperatures. This would be similar to what could occur in field soils between watermelon plantings.

Unfortunately, the *Sphaerodes* isolate was never able to be identified or recovered from plant roots by itself. This was true whether it was from roots inoculated with isolate LE, or from plants transplanted into soils previously infested with LE. It is not known how it may have provided any type of disease reduction if it was not reisolated from the

Table 5-3. Evaluation of the effects of isolate LE on plant mortality and total dry weights in combination with known wilt-suppressive and conducive soils.

Treatment	% dead plants	plant dry weight (g) ^a
Suppressive control	45.8abc ^b	0.59b ^b
Conductive control	95.8d	0.19c
Suppressive + LE	41.6abc	0.98ab
Conductive + LE	29.2a	1.29a
Suppressive +LE and 96-14	50.0abc	0.70b
Conductive + LE and 96-14	58.3bc	0.86ab
Suppressive + 96-14	33.3ab	0.79b
Conductive + 96-14	70.8cd	0.79b

^aEach value is the average of 24 plants (two plants per replication)

^bMeans followed by the same letter are not statistically different according to Duncan's multiple range test ($P = 0.01$).

plants. It was however, only identified from roots that were additionally infected with *F. oxysporum*.

Finally, the results of the FON propagule reduction test is presented in Table 5-4. It indicates that whatever adverse effects isolate LE has on *Fusarium*, they are not related to the direct attack upon chlamydospores. The colony counts for FON without isolate LE remained in the approximate range in which they were originally applied to the soils (250/g). The resulting data from soils containing both organisms do not indicate fewer propagule numbers. In fact, the colony-forming units of *Fusarium* detected from these treatments actually appear to increase (Table 5-4). The presence or absence of plants had no effect upon any of these evaluations.

Discussion

The sodium alginate pellet system for delivery of biological control agents to the infection court was originally developed for mitosporic fungi such as *Gliocladium* and *Trichoderma* (Fravel et al. 1985, Lewis and Papavizas 1985, 1987) that are commonly used for this purpose. These are fungi that produce chlamydospores and prodigious amounts of conidia. It also seems to be an adequate technique for the ascomycete, *Sphaerodes*. It was shown that activity and survivability of the fungus was retained in the pellets stored in the refrigerator for at least two months. Very early experiments tested the pellets after air drying, and found that the fungus survived and grew in culture, but no tests were conducted in the greenhouse with dried pellets.

Table 5-4. Evaluation of soils infested with *Fusarium oxysporum* f. sp. *niveum* and *Sphaerodes retispora* var. *retispora* and the reduction of *Fusarium* propagules.

Treatment ^a	Mean propagule counts ^b
FON + LE (with plants) ^c	329.20 a ^d
FON + LE (no plants)	279.96 ab
FON (with plants)	248.00 b
FON (no plants)	236.80 b

^aFON = *F. oxysporum* f. sp. *niveum* infested in soils at 250 propagules per gram of soil
LE = *S. retispora* var. *retispora* mixed into soils at 5 g pellets per 280-300 gram of soil

^bAverage number of FON colonies formed on Komada's medium after dilution plating of infested soils and 2-3 months of incubation in the greenhouse. Each value is the mean of 100 plates per treatment (10 plates per replication).

^cwatermelon seedlings were transplanted into soils and maintained and replaced as needed for the duration of the experiment

^dMeans followed by the same letter are not statistically different according to Duncan's multiple range test ($P = 0.01$).

The preliminary experiments of treating plants with the pellets and introducing the pathogen with conidial suspensions were critical for the establishment of the feasibility for the whole concept. This is an unnatural way to test pathogenicity, but it was assumed that if it worked in this manner, then the technique would more likely be successful using pathogen-infested soils. It was also noted that the treatments using pellets without inoculation with the pathogen seemed to result in larger, more vigorous plants than the untreated, healthy controls. However, whatever effect the pellets may have had nutritionally, the second group of inoculation treatments (Test 3, Table 5-1) proved that the pellets had no protective effect against FON. These treatments were not much better than the FON-inoculated controls (Table 5-1).

Because of the extremely slow growth of *Sphaerodes* in the absence of *F. oxysporum* or any of its metabolic products, another treatment was evaluated for the delivery of the potential biocontrol fungus. This involved one of the non-pathogenic isolates of *F. oxysporum*, 96-14, that was originally found in the wilt-suppressive soil in Leesburg, FL. It was chosen as the candidate for delivery for two reasons. One was that in previous tests, it showed no tendencies to induce resistance in the plants that is thought to be the source of suppressiveness in this field. Larkin et al. (1993, 1996) had shown that many of the saprophytic isolates of *F. oxysporum* from this field seemed to induce a protective resistance to plants before the pathogenic isolates could react. Others collected from this same field did not produce this type of response. The second reason for selection of this isolate was that it was one of the few non-pathogenic isolates that adequately supported growth and sporulation of the parasites being evaluated (Chapter 4).

Pathogenic isolates of *F. oxysporum* generally were better hosts for these parasites than non-pathogenic ones. The idea was to determine if the isolate LE could more effectively act in its supposedly protective role if it was already colonizing the more aggressive 96-14 isolate. Perhaps the 96-14 isolate would more effectively deliver the parasite to the infection court by competing better with the rapidly growing pathogenic *Fusarium* isolate in soils. However, it was found that this treatment did not help to reduce disease severity. Isolate LE by itself was always the more effective treatment. These tests also confirmed the 96-14 had virtually no protective effect upon watermelon plants against the pathogen. The treatment containing 96-14 by itself was always very poor, and statistically very similar to the pathogen-inoculated control in terms of both percent mortality and plant dry weights (Tables 5-1 and 5-2). None of the additional treatments including 96-14 or pellets alone had any beneficial effect upon reducing disease by FON. Therefore it was assumed that whatever protective effect being observed was due to the presence of isolate LE and not from the non-pathogenic 96-14 isolate, or from increased plant growth or added nutritional benefits via the sodium alginate pellets.

The same trends and relationships of disease protection were observed with these same treatments and the pathogen-infested soils. These experiments were designed to more accurately reflect what would be encountered in a field situation. It is interesting to note that both mixing pellets in soil and treating plants with pellets prior to transplantation were equally as effective in reducing disease (Table 5-2). Both methods were statistically better than that of method 1, which root-dipped plants with suspensions of the various treatments (data not shown). This is an important point to consider because it could give valuable information for implementing into the field. Treating plants with the parasite

prior to field introduction would probably be a more practical method of introduction. It is not known if the volume of pellets used per pot would work in the same manner in field soil, but field studies would more likely require larger quantities of inoculum. Treating plants in the transplant mix at the greenhouse level could easily be incorporated into production systems in Florida, since many watermelons are transplanted into the field. This concept could presumably be tested with other vegetables like tomatoes or peppers as well.

The use of these biocontrol treatments in conjunction with wilt-suppressive and conducive soils also gave very similar results. The conducive soil mixed with isolate LE was statistically better than any other treatment, including any other treatment involving the suppressive soil. This indicates that *Sphaerodes* may be capable of improving the benefits of the suppressive soil alone. However, since the suppressive soil itself had such a high level of disease in these evaluations, this could be a misleading result. The real test for the potential for biological control with isolate LE will need to be judged under field conditions. Successful biological control at the greenhouse level often is not continued in the field due to other interrelated environmental factors. This project has provided some evidence however, that certainly encourages the attempt to expand into field studies.

The mechanism of exactly how isolate LE is accomplishing this phenomenon is still in doubt. The ecological studies attempted here have not completely answered this question. It was firmly established that *Sphaerodes* was capable of long-term survival in soils, but it was never isolated by itself from plant roots. Plants treated with isolate LE were continually plated on media, but cultures were always overgrown with other organisms before identification could take place. Isolate LE was identified from roots

dually-inoculated with 96-14, or from roots grown in soils infested with both pelletized LE and FON.

The experiments testing the ability to reduce propagule counts indicates that fusarial chlamydospores are not directly attacked by *Sphaerodes* since the numbers were not reduced in the presence of the parasite. Although this does not explain how disease severity is seemingly reduced, it is consistent with reports of this type of parasitism. The type of parasitism among fungi like *Sphaerodes* or *Melanospora* has been assumed to occur as a fusion of hyphal strands between organisms (Jeffries and Young 1994, Hoch 1977), and not an assault on ungerminated overwintering propagules of the host. Its affect upon *F. oxysporum* may be attaching to and parasitising germ tubes and hyphae, thereby reducing vegetative growth. However, the two to three month incubation period used here may not be enough time to completely evaluate this aspect.

One of the possibilities to explain the apparent reduction of wilt disease may be that *Sphaerodes* induces some type of resistance to the plant before pathogen infection takes place. The data appear to at least suggest this, however, it is difficult to make this claim since isolate LE was never able to be reisolated from treated roots without the additional presence of *Fusarium*. Perhaps the more likely explanation is that *Sphaerodes* simply remains in a quiescent state until it senses the presence of *F. oxysporum*, and is then stimulated to begin germination and growth. This possibility was alluded to in Chapter 3 when it was determined that these parasites were very dependent upon *F. oxysporum* for some metabolic product required as a growth factor. This perhaps could explain its inability to grow and outcompete other soil fungi isolated from roots in culture in the absence of *F. oxysporum*.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Fusarium wilt has been problematic for successful crop production for over 100 years, and yet there are still very few viable options for disease management. The discovery of a group of closely related pyrenomycetous ascomycetes, currently classified in the order Sordariales, has provided the springboard for their evaluation as potential biological control agents for the wilt disease of watermelons. All six isolates tested in this study were originally found naturally occurring in association with different *formae speciales* of *Fusarium oxysporum*. The idea of mycoparasitism originated after observing the severe effects caused by these fungi on fusarial growth and vigor in culture.

Although all isolates were initially thought to be the same fungus, it was eventually determined that they actually consisted of three separate genera. This illustrates one of the problems with this group of fungi. They have all been confused with one another for many years because of superficially similar morphological characteristics, and therefore, synonyms and incorrectare replete throughout the historical literature. However, based upon the current taxonomic concept utilizing ascospore ornamentation and morphology, the six isolates were identified as *Melanospora zamiae*, *Sphaerodes retispora* var. *retispora*, and *Persiciospora moreaui*. The field isolations of these six strains were found in association with four distinct *formae speciales* from two separate states, Texas and Florida. It is also significant that four of the five from Florida were all found to be *S.*

retispora. This work also represents the first report for the discovery of *P. moreaui* in the western hemisphere. Prior to this the only two reports for this fungus have been from France and New Zealand.

An extensive amount of research on the basic biological relationships among and between these two groups of fungi have been attempted. It was shown how a strict nutritional dependency exists between the pyrenomycetes and their *Fusarium* hosts. Both growth and sporulation were significantly enhanced by the presence of either *F. oxysporum* itself, or by an unknown metabolic product obtained from the liquid culture of *Fusarium*. This compound was found to be heat stable and diffusable through dialysis tubing. When added to media, it stimulated growth and perithecial production of all pyrenomycetes in the same manner as that by *F. oxysporum*. Although this phenomenon was observed with all isolates, there were significant differences in their abilities to reproduce on the different *formae speciales*.

All six isolates were additionally determined to be parasitic on the watermelon wilt pathogen, *F. oxysporum* f. sp. *niveum* (FON). However, through series of assays, not all were found to be pathogenic to FON. *Persiciospora* did not cause measurable damage in the cultural pathogenicity assays. *Melanospora* was found to be more harmful than *Persiciospora*, but less so than *Sphaerodes*. *Melanospora* has previously been demonstrated to be parasitic on a number of fungi, including *F. oxysporum* f. sp. *lycopersici*. This work has expanded upon the *F. oxysporum* host range and more conclusively confirmed these early observations. It has further demonstrated for the first time that *Persiciospora* and *Sphaerodes* are also capable of existing in this ecological role as deleterious parasites. This study has also been unique in that it has attempted to design

and implement tests to more quantitatively measure parasitism and/or harmful effects caused by the parasites on *F. oxysporum* at the cultural level. The most effective isolate regarding perithecial production and hyphal reduction and inhibition was isolate LE. It is interesting to note that this isolate was found originally in association with FON in a watermelon wilt-suppressive soil in central Florida.

Based upon these findings, isolate LE was chosen as the candidate for disease reduction tests from watermelon wilt in the greenhouse. A number of different delivery systems for the biocontrol agent were attempted, all consisting of the encapsulation of the mycoparasite in sodium alginate pellets. Several methods did significantly reduce disease as measured by percent mortality and total plant dry weights, compared to non-treated FON controls. Their performance was generally intermediate between the healthy and FON controls, however, it did provide results that are encouraging enough to consider testing in the field. The most practical method for vegetable production in Florida would probably be to introduce the mycoparasite into the potting mix in the greenhouse and then simply transplant into field soils.

The mechanism for the apparent disease reduction is still not known for certain. It did not reduce numbers of *Fusarium* chlamydospores artificially infested into soils maintained in the greenhouse. It was not reisolated from watermelon roots by itself, so its ability to saprophytically colonize roots is still in question. However, since no adverse effects were observed on treated plants, it can at least be concluded to be non-pathogenic on watermelon. *Sphaerodes* was successfully recovered from roots or soils dually inoculated with *F. oxysporum*, and was found to survive in soils subjected to fluctuating temperature and moisture levels for at least nine months.

It is hoped that the information obtained from this project may lead to a novel and environmentally-friendly management technique for anyone having to deal with disease problems caused by *F. oxysporum*. However, before this can be accomplished, many questions unanswered in this study must still be addressed. It is probable that each potential parasite must be rigorously tested for the ability to control each desired pathogen and wilt disease. Perhaps the most important fact learned during the course of this study is that no one parasite is likely to be found which will effectively suppress wilt diseases caused by all *formae speciales* of *F. oxysporum*.

REFERENCES

- Alabouvette, C. 1986. Fusarium wilt suppressive soils from the Chateaufort region: review of a 10 year study. *Agronomie* 6: 273-284.
- Alabouvette, C. 1990. Biological control of Fusarium wilt pathogens in suppressive soils. Pages 27-43 in: *Biological control of soil-borne plant pathogens*. D. Hornby, ed. CAB International, Wallingford, UK, 479 pp.
- Alabouvette, C., Lemanceau, P., and Steinberg, C. 1996. Biological control of Fusarium wilts: opportunities for developing a commercial product. Pages 192-212 in: *Principles and practice of managing soilborne plant pathogens*. R. Hall, ed. American Phytopathological Society Press, St. Paul MN, 330 pp.
- Alexopoulos, C. J. 1962. *Introductory mycology*, 2nd ed. John Wiley, New York, 613 pp.
- Alexopoulos, C. J., Mims, C. W., and Blackwell, M. 1996. *Introductory mycology*, 4th ed. John Wiley, New York, 869 pp.
- Appel, O. and Wollenwebber, W. W. 1910. Grundlagen einer Monographie der Gattung *Fusarium*. *Kais. Biol. Anst. F. Land. U. Forstw.*, viii, pp. 1-207.
- Asthana, R. P., and Hawker, L. E. 1936. The influence of certain fungi on the sporulation of *Melanospora destruens* Shear and of some other Ascomycetes. *Ann. Bot.* 50: 323-343.
- Atkinson, G. F. 1892. Some diseases of cotton. *Alabama Agr. Exp. Sta. Bul.* 41: 5-65.
- Baker, K. F., and Cook, R. J. 1974. *Biological control of plant pathogens*. W. H. Freeman, San Francisco, 433 pp.
- Barnett, H. L., and Binder, F. L. 1973. The fungal host-parasite relationship. *Ann. Rev. Phytopathol.* 11: 273-292.
- Barr, M. E. 1990. Prodomus to nonlichenized, pyrenomycetous members of class hymenozoomycetes. *Mycotaxon* 39: 43-184.
- Bessey, E. A. 1935. *A text-book of mycology*. P. Blakiston's Sons, Philadelphia, 495 pp.

- Bessey, E. A. 1961. Morphology and taxonomy of fungi. Hafner, New York 791 pp.
- Biles, C. L., and Martyn, R. D. 1989. Local and systemic resistance induced in watermelons by *formae speciales* of *Fusarium oxysporum*. Phytopathology 79: 856-860.
- Booth, C. 1971. The genus *Fusarium*. Commonwealth Mycological Institute, Kew, Surrey, 237 pp.
- Booth, C. 1984. The *Fusarium* problem: historical, economic, and taxonomic aspects. Pages 1-13 in: The applied mycology of *Fusarium*. M. O. Moss, and J. E. Smith, eds. Cambridge University Press, Cambridge, 264 pp.
- Cannon, P. F., and Hawksworth, D. L. 1982. A re-evaluation of *Melanospora* Corda and similar pyrenomyces, with a revision of the British species. Bot. J. Lin. Soc. 84:115-160.
- Carrier, L. 1924. The beginnings of agriculture in America. McGraw-Hill, New York, 323 pp.
- Clements, F. E., and Shear, C. L. 1931. The genera of fungi. H. H. Wilson, New York, 497 pp. + 56 pl.
- Cochrane, V. W. 1958. Physiology of fungi. John Wiley and Sons, Inc. New York, 524 pp.
- Cook, R. J. 1990. Twenty-five years of progress towards biological control. Pages 11-14 in: Biological control of soil-borne plant pathogens. D. Hornby, ed. CAB International, Wallingford, UK, 479 pp.
- Cook, R. J., and Baker, K. F. 1983. The nature and practice of biological control of plant pathogens. The American Phytopathological Society, St. Paul, MN, 539 pp.
- Coons, G. H. 1928. Some aspects of the *Fusarium* problem. Pages 43-92 in: Plant pathology and physiology in relation to man. W. B. Saunders Company, Philadelphia, 207 pp.
- Corda, A. C. I. 1837. *Icones fungorum*, vol. I., J. G. Calve, Prague, 449 pp.
- Dennis, R. W. G. 1978. British ascomycetes. J. Cramer, Lehre, Germany, 585 pp.
- Doguet, G. 1955. Le genre *Melanospora*. Le Botaniste 39: 1-313.

- Eastcott, E. V. 1928. Wildier's bios. The isolation and identification of "bios I." J. Phys. Chem. 32: 1094-1111.
- Esposito, R., and Fletcher, A. M. 1961. The relationship of pteridine biosynthesis to the action of copper 8-hydroxyl-quinolate of fungal spores. Arch. Biochem. Biophys. 93: 369-376.
- Fitzpatrick, H. M. 1934. Lectures on mycology, general introduction and ascomycetes. Plant Pathology Dept., Cornell University, Ithaca, New York, 314 pp.
- Fravel, D. R., Marois, J. J., Lumsden, R. D., and Connick, W. J. 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. Phytopathology 75: 774-777.
- Gessler, C., and Kuc, J. 1982. Induction of resistance to *Fusarium* wilt in cucumber by root and foliar pathogens. Phytopathology 72: 1439-1441.
- Gilmer, J. C. 1957. A manual of soil fungi. Iowa State College Press. Ames, IA, 450 pp.
- Goh, T.-K., and Hanlin, R. T. 1994. Ascomal development in *Melanospora zamiae*. Mycologia 86: 357-370.
- Griffin, D. H. 1981. Fungal physiology. John Wiley and Sons, New York, 383 pp.
- Gwynne-Vaughn, H. C.I., and Barnes, B. 1927. The structure and development of the fungi. University Press, Cambridge, 384 pp.
- Hanlin, R. T., Goh, T.-K., and Tortolero, O. 1993. Icones ascomycetum venezuelae: *Melanospora zamiae*. Mycotaxon 49: 167-174.
- Harveson, R. M., and Rush, C. M. 1997. Genetic variation among *Fusarium oxysporum* isolates from sugar beet as determined by vegetative compatibility. Plant Dis. 81: 85-88.
- Harveson, R. M., and Kimbrough, J. W. 1998. Evaluation of the parasitic relationship between *Melanospora* spp. and *Fusarium oxysporum*. Phytopathology 88: S36.
- Hawker, L. E. 1936. The effect of certain accessory growth substances on the sporulation of *Melanospora destruens* and of some other fungi. Ann. Bot. 50: 699-717.
- Hawker, L. E. 1938. Effect of growth substances on growth and fruiting of *Melanospora destruens*. Nature 142: 1038.

- Hawker, L. E. 1939. The nature of the accessory growth factors influencing growth and fruiting of *Melanospora destruens* Shear. And of some other fungi. *Ann. Bot. N.S.* 3:657-675.
- Hawker, L. E. 1950. *Physiology of fungi*. University of London Press, London, 360 pp.
- Hawker, L. E. 1951. Morphological and physiological studies on *Sordaria destruens* (Shear) comb. nov. (syn. *Melanospira destruens*), *Sordaria fimicola* and *Melanospora zamiae*. *Trans. Brit. Mycol. Soc.* 34: 174-186.
- Hawksworth, D. L., Kirk, P. M., Sutton, B. C., and Pegler, D. N. 1995. *Dictionary of the fungi*. CAB International, Wallingford, UK. 616 pp.
- Hawksworth, D. L., and Udagawa, S. 1977. Contributions to a monograph of *Microthecium*. *Trans. Mycol. Soc. of Japan.* 18: 143-154.
- Heald, F. D., and Poole, V. W. 1909. The influence of chemical stimulation upon the production of perithecia of *Melanospora pampeana* Speg. *Nebr. Agr. Exp. Sta. Rept.* 22: 130-134.
- Higgins, B. B. 1909. Is *Neocosmospora vasinfecta* (Atk) Smith, the perithecial stage of *Fusarium* which causes cowpea wilt? *North Carolina Agr. Exp. Sta. 32nd Ann. Rep.*: 100-116.
- Hoch, H. C. 1977. Mycoparasitic relationships: *Gonatobotrys simplex* parasitic on *Alternaria tenuis*. *Phytopathology* 67: 309-314.
- Hopkins, D. L., and Elmstrom, G. W. 1976. Effect of soil pH and nitrogen source on *Fusarium* wilt of watermelon on land previously cropped to watermelons. *Proc. Fla. State Hort. Soc.* 89: 141-143.
- Hopkins, D. L., and Elmstrom, G. W. 1979. Evaluation of soil fumigants and application methods for control of *Fusarium* wilt of watermelon. *Plant Dis. Rep.* 63: 1003-1006.
- Hopkins, D. L., and Elmstrom, G. W. 1984. *Fusarium* wilt in watermelon cultivars grown in a 4-year monoculture. *Plant Dis.* 68: 129-131.
- Hopkins, D. L., and Elmstrom, G. W. 1987. Cultivar-specific induction of soil suppressiveness to *Fusarium* wilt of watermelon. *Phytopathology* 77: 607-611.
- Horie, Y., Udagawa, S., and Cannon, P. F. 1986. Four new Japanese species of the Ceratostomataceae (Ascomycetes). *Mycotaxon* 25: 229-245.

- Jeffries, P., and Young, T. W. K. 1994. Interfungal parasitic relationships. CAB International, Wallingford, UK 296 pp.
- Jones, J. P., Engelhard, A. W., and Woltz, S. S. 1989. Management of *Fusarium* wilt of vegetable and ornamentals by macro- and micronutrient nutrition. Pages 18-32 in: Soilborne pathogens: management of diseases with micro- and macroelements. American Phytopathological Society, St. Paul MN, 217 pp.
- Jones, J. P., and Woltz, S. S. 1981. *Fusarium*-incited diseases of tomato and potato and their control. Pages 157-168 in: *Fusarium*: Diseases, biology, and taxonomy. P. E. Nelson, T. A. Toussoun, and R. J. Cook, eds. Penn State University Press, University Park, PA, 457 pp.
- Jones, K. G., and Blackwell, M. 1998. Phylogenetic analysis of ambrosial species in the genus *Raffaella* based on 18S rDNA sequences. Mycol. Res. 102: 661-665.
- Jordan, E. G., and Barnett, H. L. 1978. Nutrition and parasitism of *Melanospora zamiae*. Mycologia. 70: 300-312.
- Komada, 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Plant Prot. Res. 8: 114-125.
- Krug, J. C. 1988. A new species of *Persicospora* from African soil. Mycologia 80: 414-417.
- Kuc, J. 1982. Induced immunity to plant diseases. BioScience 32: 854-860.
- Kuc, J. 1990. Immunization for the control of plant diseases. Pages 355-373 in: Biological control of soil-borne plant pathogens. D. Hornby, ed. CAB International, Wallingford, UK, 479 pp.
- Larkin, R. P., Hopkins, D. L., and Martin, F. N. 1993. Ecology of *Fusarium oxysporum* f. sp. *niveum* in soils suppressive and conducive to *Fusarium* wilt of watermelon. Phytopathology 83: 1105-1116.
- Larkin, R. P., Hopkins, D. L., and Martin, F. N. 1996. Suppression of *Fusarium* wilt of watermelon by nonpathogenic *Fusarium oxysporum* and other microorganisms recovered from a disease-suppressive soil. Phytopathology 86: 812-819.
- Lewis, J. A., and Papavizas, G. C. 1985. Characteristics of alginate pellets formulated with *Trichoderma* and *Gliocladium* and their effect on the proliferation of the fungi in soil. Plant Path. 34: 571-577.
- Lewis, J. A., and Papavizas, G. C. 1987. Application of *Trichoderma* and *Gliocladium* in alginate pellets for control of *Rhizoctonia* damping-off. Plant Path. 36: 438-446.

- Livingstone, D. 1859. Missionary travels and researches in South Africa. Harper, New York, 755 pp.
- Luttrell, E. S. 1951. Taxonomy of the pyrenomycetes. University of Missouri Stud. Sci. Ser. 24 (3): 1-120.
- Malloch, D. 1970. The genera of cleistothecial ascomycota. Ph.D. dissertation, University of Toronto, 672 pp.
- Malloch, D. 1976. *Scopinella sphaerophila*. Fungi Candenses No. 83.
- Marois, J. J., and Mitchell, D. J. 1981. Effects of fumigation and fungal antagonists on the relationships of inoculum density to infection incidence and disease severity to Fusarium crown rot of tomato. Phytopathology 71: 167-170.
- Martyn, R. D. 1986. Use of soil solarization to control Fusarium wilt of watermelon. Plant Dis. 70: 762-766.
- Martyn, R. D. 1996. Fusarium wilt of watermelon. Pages 13-14 in: Compendium of cucurbit diseases. T. A. Zitter, D. L. Hopkins, and C. E. Thomas, eds. American Phytopathological Society, St. Paul, MN, 134 pp.
- Molliard, M. 1903. Role des bacteries dans la production des peritheces des Ascobolus. Comp. Rend. Acad. Sci. (Paris) 136: 899-901.
- Munk, A. 1957. Danish pyrenomycetes. A preliminary flora. Dansk. Bot. Ark. 17: 1-491.
- Nannfeldt, J. A. 1932. Studien uber die morphologie und systematik der nicht-lichenisierten inoperculaten discomyceten. Nova Acta Regiae Soc.Sci. Upsal. IV 8: 1-368.
- Nelson, P. E. 1981. Life cycle and epidemiology of *Fusarium oxysporum*. Pages 51-80 in: Fungal wilt diseases of plants. M. E. Mace, A. A. Bell, and C. H. Beckman, eds. Academic Press, New York, 640 pp.
- Ogawa, K., and Komada, H. 1985. Biological control of Fusarium wilt of sweet potato with cross-protection by nonpathogenic *Fusarium oxysporum*. Pages 121-124 in: Ecology and management of soilborne pathogens. C. A. Parker, A. D. Rovira, K. J. Moore, P. T. W. Wong, and J. F. Kollmorgen, eds. American Phytopathological Society, St. Paul, MN, 358 pp.
- Orton, W. A. 1907. A study of disease resistance in watermelons. Science 25: 288.

- Orton, W. A. 1909. The development of farm crops resistant to disease. United States Department of Agriculture Yearbook, 1908: 453-464.
- Paulitz, T. C., Park, C. S., and Baker, R. 1987. Biological control of *Fusarium* wilt of cucumber with nonpathogenic isolates of *Fusarium oxysporum*. Can. J. Microbiol. 33: 349-353.
- Peirce, L. C. 1987. Vegetables, characteristics production, and marketing. New York, 433 pp.
- Phillips, D. V., Leben, C., and Allison, C. C. 1967. A mechanism for the reduction of *Fusarium* wilt by a *Cephalosporium* species. Phytopathology 57: 9166-919.
- Porter, D. R., and Melhus, I. A. 1932. The pathogenicity of *Fusarium nivium* (EFS.) and the development of wilt resistant strains of *Citrullus vulgaris* (Schrad.) Iowa Agr. Exp. Sta. Bul. 149: 121-184.
- Price, D. 1984. *Fusarium* and plant pathology: the reservoir of infection. Pages 71-93 in: The applied mycology of *Fusarium*. M. O. Moss, and J. E. Smith, eds. Cambridge University Press, Cambridge, 264 pp.
- Rehner, S. A., and Samuels, G. J. 1995. Molecular systematics of the hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. Can. J. Bot. 73: S816-S823.
- Reinking, O. H., and Manns, M. M. 1933. Parasitic and other fusaria counted in tropical soils. Zeitschr. Parasitenk 6: 23-75.
- Robbins, W. J., and Kavanagh, F. 1938. Vitamin B₁ or its intermediates and growth of certain fungi. Am. J. Bot. 25: 229-236.
- Sauer, J. D. 1994. Historical geography of crop plants. CRC Press, Boca Raton, FL, 309 pp.
- Scher, F. M., and Baker, R. 1980. Mechanism of biological control in a *Fusarium*-suppressive soil. Phytopathology 70: 412-417.
- Schneider, R. W. 1984. Effect of nonpathogenic strains of *Fusarium oxysporum* on celery root infection by *F. oxysporum* f. sp. *apli* and a novel use of the Lineweaver-Burk double reciprocal plot technique. Phytopathology 74: 646-653.
- Shear, C. L., and Dodge, B. O. 1927. Life histories and heterothallism of the red bread-mold fungi of the *Monilia sitophila* group. J. Agr. Res. 34: 1019-1042.

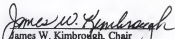
- Shimotsuma, M., Kuc, J., and Jones, C. M. 1972. The effects of prior inoculations with non-pathogenic fungi on *Fusarium* wilt of watermelon. *HortScience* 7: 72-73.
- Singleton, P., and Sainsbury, D. 1987. Dictionary of microbiology and molecular biology. John Wiley, New York. 1019 pp.
- Smith, E. F. 1894. The watermelon disease of the South. *American Assoc. Adv. Sci. Proc.* 43: 289-290.
- Smith, E. F. 1899. Wilt diseases of cotton, watermelon, and cowpea. United States Department of Agriculture, Div. of Veg. Path. and Phys. Bul. 17: 7-73.
- Smith, S. N., and Snyder, W. C. 1971. Relationship of inoculum density and soil types to severity of *Fusarium* wilt of sweet potato. *Phytopathology* 61: 1049-1051.
- Snyder, W. C. and Hansen, H. N. 1940. The species concept in *Fusarium*. *Am J. Bot.* 27: 64-67.
- Spatafora, J. W., and Blackwell, M. 1994. Cladistic analysis of partial *ssrDNA* sequences among unitunicate perithecial ascomycetes and its implications on the evolution of centrum development. Pages 233-241 in: *Ascomycete systematics, problems and perspectives in the nineties*. D. L. Hawksworth, ed. Plenum Press, New York, 453 pp.
- Stevens, R. B. 1974. *Mycology guidebook*. University of Washington Press, Seattle. 703 pp.
- Taubenhaus, J. J. 1920. Wilts of watermelon and related crops. *Texas Agr. Exp. Sta. Bul* 260: 1-50.
- Toussoun, T. A. 1975. *Fusarium*-suppressive soils. Pages 145-151 in: *Biology and control of soil-borne plant pathogens*. G. W. Bruehl, ed. American Phytopathological Society, St. Paul MN, 216 pp.
- Tsuneda, A., Malloch, D., and Hiratsuka, Y. 1985. Ascospore morphology and germination in *Scopinella* species. *Trans. Mycol. Soc. Japan* 26: 221-229.
- Udagawa, S., and Cain, R. F. 1969. Notes on the genus *Microthecium*. *Can. J. Bot.* 47: 1915-1933.
- Vakili, N. G. 1989. *Gonatobotrys simplex* and its teleomorph, *Melanospora damnosus*. *Mycol. Res.* 93: 67-74.
- Von Arx, J. A. 1981. The genera of fungi sporulating in pure culture. J. Cramer, Lehre, Germany. 315 pp.

- Walker, J. C. 1957. Plant pathology. McGraw-Hill Book Company Inc., New York, 707 pp.
- Walker, J. C., and Snyder, W. C. 1933. Pea wilts and root rots. Wisconsin Agr. Exp. Sta. Bul. 424: 1-16.
- Watanabe, T. 1997. Stimulation of perithecium and ascospore production in *Sordaria fimicola* by *Armillaria* and various fungal species. Mycol. Res. 101: 1190-1194.
- Wehmeyer, L. E. 1975. The pyrenomyceous fungi. Mycol. Memoir No. 6. J. Cramer, Lehre, Germany. 250 pp.
- Whaley, J. W., and Barnett, H. L. 1963. Parasitism and nutrition of *Gonatobotrys simplex*. Mycologia 55: 199-210.
- Wollenwebber, W. W. 1913. Studies on the *Fusarium* problem. Phytopathology 3: 24-50.
- Wollenwebber, W. W., and Reinking, O. A. 1935. Die Fusarien, ihre Beschreibung, Schadwirkung, und Bekämpfung. Paul Parey, Berlin. 350 pp.
- Wymore, L. A., and Baker, R. 1982. Factors affecting cross-protection in control of *Fusarium* wilt of tomato. Plant Dis. 66: 908-910.

BIOGRAPHICAL SKETCH

Robert M. Harveson was born in Abilene, TX, on October 17, 1960. He graduated from Bridgeport High School in Bridgeport, TX, in 1979 as salutatorian. He received a B.A. in history from Trinity University in San Antonio, TX, in May 1983. He then obtained a B.S. degree from Tarleton State University in plant and soil science in May 1985, and graduated magna cum laude. After working for a year, he began an M.S. in plant pathology as a graduate teaching assistant at Texas A&M University. He finished his project working with virus diseases of cucurbits and graduated in May 1989. He then was hired in June 1989 by the University of Florida as a diagnostician to start and manage a new satellite plant disease diagnostic clinic located at the Southwest Florida Research and Education Center in Immokalee, FL. After two years, he relocated to Amarillo, TX, and worked for five years as a research associate for Texas A&M, conducting research on soilborne viral and fungal diseases of sugar beets and wheat. In January 1996, he started a Ph.D. program at the University of Florida, and will graduate in August 1999. He has accepted a 50% research-50% extension position as an assistant professor of plant pathology with the University of Nebraska. He will be located at the Panhandle Research and Extension Center in Scottsbluff, NE, and be responsible for diseases of sugar beets, dry-edible beans, and potatoes beginning in July 1999. He married Tammy Renee Phylar on June 5, 1999.

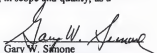
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James W. Kimbrough, Chair
Professor of Plant Pathology

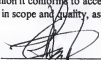
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Donald L. Hopkins, Cochair
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1999



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